THE RELATIONSHIP BETWEEN RESISTANCE TO THE FUNGAL TOXIN CERCOSPORIN AND GREY LEAF SPOT DISEASE RESISTANCE IN CORN (ZEA MAYS L.)

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by

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An abstract of a Thesis by
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The problem. Corn is infected by the fungus Cerospora zeae-maydis which causes the disease Grey Leaf Spot. The fungus produces a toxin, cercosporin, whose role in the pathogenicity of the disease is not known. The first step in determining the importance of the toxin to the fungus is to screen corn inbreds to see if any disease resistant inbreds are also resistant to the toxin.

Procedure. Thirteen corn inbreds were tested for their degree of resistance to the fungal toxin cercosporin. The level of tolerance to cercosporin for each inbred was then compared to the inbred's level of resistance to Grey Leaf Spot. Resistance to cercosporin was determined by measuring the increases in the conductivity of water containing leaf tissue slices following exposure to cercosporin. Ion leakage is an indicator of cellular damage, so resistant tissue would be expected to have a lower level of conductivity increase than susceptible tissue. Resistance to Grey Leaf Spot disease had been previously determined by field test conducted by Pioneer Hi-Bred International, Inc.

Findings. The ion leakage assay showed that the inbreds which were more disease resistant tended to have a lower tolerance for cercosporin than did the less disease resistant inbreds (correlation coefficient = 0.5524).

Conclusion. Based on the lack of a positive relationship between disease susceptibility and cercosporin susceptibility it was concluded that cercosporin resistance does not have a significant role in the prevention of Grey Leaf Spot infections among the tested inbreds.

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INTRODUCTION

Plants are affected by a wide variety of diseases ranging in severity from mild to lethal. When these diseases infect cash crops such as corn, soybeans, or tobacco, the economic effects can be devastating. It is therefore, important to understand the way plant pathogens cause diseases in order to try to prevent infection either through protective measures or breeding for resistance.

Within the fungal genus Cercospora are a number of plant pathogens that cause leaf spot diseases on crop species. One such disease is Grey Leaf Spot of corn caused by C. zeae-maydis. The disease was first reported in Alexander County, Illinois in 1924. Grey Leaf Spot has increased in intensity with the increase in the practice of minimal tillage farming and has spread from the mid-Atlantic and southeastern regions of the United States to the Midwest (6,21). The fungus infects corn leaves under conditions of cool temperatures and relatively high humidity. Once an infection occurs lesions begin to appear on the leaves of the infected plants. In corn (C. zeae-maydis), lesions require two to three weeks for maturation and are 1-6 cm long and 2-4 mm wide. The lesions first appear tan in color, but as sporulation becomes dense the lesions take on a grayish cast, hence the name Grey Leaf Spot. A yellow halo may appear around immature lesions when they are observed by transmitted light. As the lesions mature they become opaque with a well defined rectangular border. With progression of the disease, upwards of 90% of the photosynthetic surface of the leaves can be covered by lesions. At this

point severe water loss occurs and the pathogen can spread to the stalk causing lodging and severe crop loss (21).

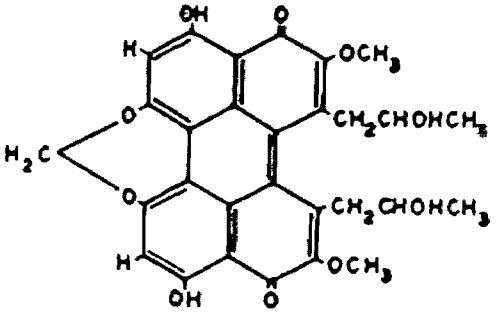
In contrast, lesions produced by Cercospora nicotianae on tobacco are irregularly circular and as large as 10 mm in diameter. The lesions have a tannish gray or white center with a brown margin and a surrounding region of chlorotic tissue. It takes six to ten days for the lesions to develop (30).

In addition to producing a wide range of lesion types, Cercospora species range considerably in morphology. One thing, however, that the members of the genus have in common is the production of the pigment cercosporin. cercosporin is suspected to play a role in the pathogenicity of several Cercospora species. In one study (31) the toxin was extracted from C. beticola, a pathogen of sugar beet, and applied by microsyringes in one microliter droplets to the adaxial surface of Beta vulgaris (sugar beet) leaves. The lesions produced were more irregular than those produced by an actual infection, but were similar in size and appearance. When these lesions were examined more closely by electron microscopy, the cells at the center of the lesion appeared collapsed and filled by electron-dense cytoplasmic remains. In cells farther away from the center of the lesion the degree of collapse and necrosis became less severe. Cell walls tended to remain undamaged, yet the nuclear membrane, tonoplast, plasmalemma, chloroplast outer membrane, and stroma were fragmented or disintegrated. In addition, the mitochondria's cristae were greatly reduced and indistinct. These cercosporin-induced lesions observed at the ultrastructural level were similar, but not identical, to C. beticola-induced lesions (31).

Cercosporin is a non-host specific toxin in the sense that it can cause cellular damage to most plant species tested (11,13,24). Cercosporin has been isolated from the following Cercospora species which infect a variety of host species: apii, ariminiensis, bertoreae, beticola, bizzozeriana, canescens, carotae, chenopodii, cistinearum, citrullina, cladosporioides, columaris, diazu, erysimi, ferruginea, fusca, hayii, kikuchii, malvacearum, malvicola, medicaginis, microsora, nicotianae, oryzae, personata, plantaginis, ricinella, rosicola, rubi, setariae, smilacis, unamunoi, violae, zeae-maydis, and zinniae (2,3,13,27,32). Some species of Cercospora have been identified which do not produce the toxin. These species, however, are considered by some not to be true members of the genus (13).

Cercosporin was first isolated from Cercospora kikuchii, a pathogen of soybeans, by ether extraction. A molecular formula of $C_{30}H_{28}O_{10}$ (M.W. 550-560) was suggested (20) but was later proved incorrect.

The actual structure of cercosporin was independently reported by two groups (23,33).



The structure was determined from mass spectroscopy and NMR spectral data. Cercosporin has a molecular weight of 534 and a chemical formula of

$C_{29}H_{26}O_{10}$, and has the formula name [1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone] (10).

An important characteristic of the toxin is that it is photodynamic. The toxin is active in producing cellular damage when exposed to light in the range of 400 to 600 nm. The action spectrum for damage caused by cercosporin matches that of the absorbance spectrum of cercosporin within this range, with a maximum at around 473 nm (11). On the other hand, cercosporin is also unstable in the light as it is photodegraded rapidly in an aqueous system. Cercosporin also isomerizes to isocercosporin, a conformer produced by a shift in bond angles in the conjugated ring, when exposed to temperatures above 50^{o} C (33).

Cercosporin's toxicity is due to the production of toxic forms of oxygen. When cercosporin is exposed to light of the appropriate wavelength it produces both superoxide radicals and singlet oxygen (9). Cercosporin is capable of killing both mice and bacteria as well as causing ion leakage from such tissues as corn, carrot, beet, and potato when exposed to light and in the presence of oxygen (11).

Ground state dioxygen (O_2) is much less reactive than expected as it contains two unpaired electronic spins making it paramagnetic; therefore, addition of a pair of electrons is in violation of the Pauli Exclusion Principle. In order for ground state dioxygen to react with non-radical substances a spin inversion must take place, but this is a rather slow process creating a barrier to non-radical reactions. One way to make oxygen more reactive is to excite dioxygen to the diamagnetic singlet state, thus circumventing the spin restrictions and creating singlet oxygen ($^1O_2$). Another way to increase

dioxygen's reactivity is to add electrons to it one at a time allowing time for spin inversions to take place. This method produces the intermediate superoxide ($O_2^{\cdot -}$) with the addition of a single electron (28).

Cercosporin is thought to absorb light forming a long lived electronically excited state or triplet state. This excited state (triplet sensitizer (3S)) can react with oxygen by one of two pathways forming either singlet oxygen or superoxide. The first pathway leads to the production of singlet oxygen when the triplet sensitizer reacts directly with oxygen itself exciting it to the diamagnetic state.

$$^3S \xrightarrow{O_2} {}^1O_2 + S$$

The second pathway involves a reducing substrate (R or RH) through which either an electron or a hydrogen atom is transferred to cercosporin. The resulting sensitizer radical reacts with oxygen giving it an electron to produce superoxide radical (9).

$$R^{\cdot +} + S^{\cdot -} \xleftarrow{R} {}^3S \xrightarrow{RH} SH^{\cdot} + R^{\cdot}$$

then

$$S^{\cdot -} \text{ or } SH^{\cdot} \xrightarrow{O_2} O_2^{\cdot -} \text{ or } HO_2^{\cdot} + S$$

It is the production of these oxygen compounds that is of particular importance. Singlet oxygen reacts electrophilically with compounds containing multiple double bonds such as certain phospholipids found in membranes (19). The reaction of singlet oxygen with polyunsaturated fats

produces lipid peroxides which, once formed, can continue to regenerate, thus self-catalyzing the process of fatty acid autoxidation (29). This process eventually leads to the complete breakdown of fatty acids and lipids causing severe damage to the cell's enzymes and membranes, leading to a loss in electrical resistance and membrane integrity (17). Superoxide, the other toxic form of oxygen created by cercosporin, is not very reactive. For instance, unlike singlet oxygen, superoxide radicals cannot directly react with membrane lipids to cause peroxidation. There is evidence, however, that superoxide radicals can react with hydrogen peroxide in the presence of ferrous iron to form hydroxyl radicals, which are extremely reactive. Hydroxyl radicals are capable of causing lipid peroxidation and consequently fatty acid autoxidation (17).

Experiments have provided evidence that these radicals are responsible for the killing of cells by cercosporin (11). When suspension-cultured tobacco cells were exposed to cercosporin at concentrations of 0.2 and 5 uM and incubated in the light, all the cells were killed within 48 and 4 hours respectively. When not exposed to light the cells survived in toxin concentrations as high as 40 uM for as long as seven days. The same results were obtained using sugar beet suspension-culture cells. In other experiments, two different singlet oxygen quenchers (bixin and DABCO) were added to the cultures and both delayed killing of the cells. After a lag time, however, cell death proceeded at a rate similar to controls. It is assumed that killing proceeds after a lag time as the quenching agents are consumed. These results strongly suggest that singlet oxygen plays a major role in the toxicity

of cercosporin. Further tests should be conducted using superoxide quenchers.

A complicating factor in understanding cercosporin's toxicity to cells is the effect of toxin on cells in the dark. It has been noted that non-irradiated cercosporin has an effect on ion transport in the cells of sugar beet leaves (25). The authors proposed that the effect could be caused by a direct interaction of cercosporin with plasmalemma ATPases or mitochondria (25). Either way, cercosporin apparently can damage cells even if not exposed to light. This data is puzzling in the light of earlier studies with tobacco leaf tissue which showed there to be no significant cell damage caused by cercosporin in the absence of light (10). More study is needed to understand cercosporin's effects on cells in the absence of light.

Although cercosporin is a non-host specific toxin, several potential mechanisms exist by which cells could resist the toxin. Elevated levels of superoxide dismutase, an enzyme that functions as a superoxide quencher, could enhance the level of cercosporin resistance. This type of resistance was first noted in tobacco callus selected for resistance to the superoxide radical-producing herbicide paraquat [1,1'-dimethyl-4,4'-bipyridylium salt] (18). It was observed that in resistant tobacco callus lines there was a 14- to 159-fold increase in the activity of superoxide dismutase compared to leaf cells on a protein basis (15). It was demonstrated that the basis for paraquat resistance was genetic as it was passed on from callus to regenerated plants and then to their progeny. Furthermore, the plants which were paraquat resistant were partially resistant to cercosporin (18). The partial resistance of the plants to cercosporin could be due to the plants being able to dismutate the superoxide

radicals produced by the toxin. There is no evidence, however, that these plants have an enhanced defense mechanism against the singlet oxygen produced by cercosporin.

Besides superoxide dismutase, increased levels of glutathione reductase may have a possible role in toxin resistance since glutathione reductase is able to protect a cell from toxic oxygen radicals. When maize tissue is incubated in an atmosphere with increased levels of oxygen which leads to an increase in the levels of superoxide in the cell, the level of superoxide dismutase activity remains the same. The activity of glutathione reductase, however, shows a two- to three-fold increase (14). Glutathione in a reduced form is able to react with hydroxyl radicals and singlet oxygen very rapidly, helping to protect the cell. Therefore, in the presence of these radicals, increased glutathione reductase would be expected in order to return oxidized glutathione to a reduced form so it can continue to react with oxygen radicals (17). Direct tests to support the idea of glutathione reductase involvement in cercosporin resistance have not yet been carried out.

Another form of defense against cercosporin may be carotenoids which are singlet oxygen quenchers, but no evidence for this has been obtained in plants. The Cercospora species themselves, on the other hand, may utilize carotenoids as a defense mechanism against cercosporin. It has been demonstrated that carotenoid minus mutants of Neurospora crassa are more sensitive to cercosporin than wild types (8). So far, however, this same correlation has not been proven for Cercospora species, for which carotenoid minus mutants do not exist. Resistance to cercosporin by Cercospora species has also been associated with some factor in the fungal cell wall. C. nicotianae

protoplasts are vulnerable to the effects of the toxin, yet become resistant to cercosporin with the regeneration of cell wall glucans (16). It has also been demonstrated that other deuteromycete species (8,13) and ascomycete species are resistant to cercosporin, but that oomycete species are not (8). The deuteromycetes and ascomycetes have cell walls composed of chitin plus beta-glucan, whereas the oomycetes' cell walls are composed of cellulose plus beta-glucan (1). Whether it is this difference in structure that determines resistance or some other compound that is produced in deuteromycetes and ascomycetes cell walls is not known.

Attempts have been made to identify plant species which exhibit a lower susceptibility to cercosporin. In one study, detached or intact leaves were tested for toxin resistance by either infiltrating the toxin under pressure or injecting the toxin into the leaf and then monitoring the development of lesions. Of the six plant species tested, castor bean, soybean, tobacco, common bean, cow-pea, and Manihot esculenta, only M. esculenta showed a resistance to the toxin for all the concentrations tested (13). In another study, no significant difference was found in the level of toxin resistance among the four plant species tested: potato, corn, carrot, and red beet. In this study toxin resistance was determined by measuring the amount of ion leakage from disks of plant tissue floated in a solution containing cercosporin (24).

Work has also been done to test for toxin resistance among differing varieties of the same plant species. It was determined by the development of lesions on the surface of leaves inoculated with cercosporin that some strains of peanut (Arachis hypogaea L.) showed lower toxin susceptibility than others. (Melouk, unpublished). Several peanut varieties which showed this lower

toxin susceptibility were also resistant to Early Leaf Spot of peanut, caused by C. arachidicola. However, when a wider range of varieties were tested, no consistent correlation could be obtained between toxin susceptibility and disease susceptibility (Melouk, personal communication).

So far the evidence indicates that cercosporin is important in some phase of the Cercospora species' life cycle. To start with, true members of the genus, many of which are pathogens, produce cercosporin (13). Therefore, it would be reasonable to believe that cercosporin's importance is in the pathogenicity of the fungus. Cercosporin's mode of action also supports this idea. Cercosporin has been shown to produce both singlet oxygen and superoxide radicals which are in turn toxic to cells. These compounds act either directly or indirectly on cell membranes causing lipid peroxidation which ultimately leads to the destruction of the membrane's integrity. Electron micrographs have shown that there is extensive damage to the membranes of cells destroyed by cercosporin (31). Furthermore, the cercosporin-induced damage to the cells is similar to the cellular damage caused by Cercospora diseases. This is true on the macroscopic level also as the lesions produced by fungal infection and those produced by application of the toxin itself are similar (31). It may be that the fungus produces cercosporin in order to destroy the cells' membranes allowing the cell's contents to leak out into the surrounding environment. Once free of the cell any nutrients or metabolic products can be taken up and utilized by the fungus. This is important since the fungus can not actually penetrate an intact cell by itself (6).

If cercosporin actually is involved in the pathogenicity of Cercospora species such as C. zeae-maydis disease on corn, then it is reasonable to assume that cercosporin resistance in the host would impart a degree of disease resistance. This type of toxin disease relationship has been proven for several other fungal pathogens of corn in which host genes for toxin resistance are effective in reducing the severity of disease. For example, Helminthosprorium maydis race T severely infects corn lines with Texas male sterile cytoplasm. Resistance to the disease is conferred by other cytoplasms which apparently are unaffected by the toxin due to a lack of a toxin sensitive site in the mitochondria (26). A second disease showing a relationship between toxin and disease susceptibility is Leaf Spot of Corn, caused by Helminthosporium carbonum race 1. A single dominant nuclear gene, Hm1, confers resistance to both toxin and pathogen (7). These toxins, unlike cercosporin, are host-selective toxins whose role in disease is easier to define since toxin-resistant lines are available. With non-host specific toxins, like cercosporin, it is more difficult to determine how important the toxin is in disease development.

Objectives. The first step to developing a toxin based model of disease resistance for Grey Leaf Spot is to determine whether corn inbred lines which are resistant to the disease show a corresponding increase in resistance to cercosporin when compared to Grey Leaf Spot susceptible lines. Grey Leaf Spot Disease resistance is scored at Pioneer on a scale from 1.0 to 9.0 with 1.0 being the most susceptible and 9.0 being the most resistant. Therefore, if cercosporin resistance plays a role in preventing infection in resistant lines, a correlation should be evident between Grey Leaf Spot resistance score and

varying sources, it is possible that only one or a few lines employ toxin resistance as a means of tolerating the pathogen, whereas other lines prevent pathogen spread by other mechanisms. In these cases no clear-cut correlation would be seen, but one or more lines should be significantly more resistant to the toxin.

My objectives were to:

1) Develop an assay to measure the sensitivity of corn inbreds to cercosporin.

2) Measure and compare cercosporin resistance of thirteen corn inbreds to determine if there is a relationship between cercosporin resistance and Grey Leaf Spot disease resistance.

MATERIALS AND METHODS

Plant material. Maize seed was obtained for thirteen inbreds with varying degrees of resistance to Grey Leaf Spot based on Pioneer field evaluations (Table 1). Inbred lines NC250a and TZ70 were obtained from Dr. Major Goodman, Department of Crop Sciences, North Carolina State University, Raleigh, NC 27650. The rest of the inbreds were supplied by Pioneer Hi-Bred International Inc., Johnston, IA 50131. Plants were grown in twelve inch pots containing a mixture of soil, vermiculite, and perlite. Plants were fertilized once a week with Peter's Professional Fertilizer containing 20% nitrogen, 20% phosphate, and 20% potash. The plants were grown in a greenhouse under ambient light, with supplemental illumination during winter months from a

Cercosporin extraction and purification. Cercospora beticola was grown on malt agar (11) for 30 days under continuous fluorescent illumination at room temperature. After 30 days the mycelial mat and agar were blotted on paper towels and then allowed to air dry. The dried mycelia were stored in the freezer at -20° C until needed.

For toxin extraction the mycelium was ground in liquidnitrogen to a fine powder using a mortar and pestle and placed in a cellulose extraction thimble. The thimble was inserted into a Soxhlet apparatus and the pigments were extracted with diethyl ether for eight hours. The extract was air dried, reconstituted in a minimal amount of chloroform, and mixed with three grams of a treated silica gel and allowed to air dry. Silica gel was treated by mixing eight grams of phosphoric acid, sixteen grams of $Ca(H_2PO_4)_2 \cdot H_2O$ and 160 grams of LPS-2 media (Whatman) in 400 ml of distilled water. The slurry was placed in an oven at 100° C and dried overnight. After cooling, the mixture was homogenized with a mortar and pestle and placed in a flashchromatography column (T.J. Baker). The pigments mixed with silica were placed on the top of the dry column and eluted with ethyl acetate:benzene (2:3) (4). The red cercosporin band was collected, dried, and run through a recrystallization series of n-pentane:chloroform (5:1), n-pentane:chloroform (10:1), and n-hexane:chloroform (8:1). The recrystallizations were done in the freezer at -4° C (22). Purity of the sample was checked on TLC plates pre-sprayed with 2% oxalic acid and run in ethyl acetate:benzene (2:3), and by HPLC using a Waters 3.9 X 200 mm Microbondapak phenyl steel column eluted with 30% water, 70% methanol, and 0.1% TFA

Table 1. Inbreds and Grey Leaf Spot scores

Inbred	Source	Number	GLS Score
814*	Pioneer	1	2.0**
RK1*	"	2	7.3
FRMO17	"	3	2.5
RK2*	"	4	6.0
RK3*	"	5	7.0
G35*	"	6	3.0
B73	"	7	3.0
TZ70	Dr. Goodman	8	6.5
NC250a	"	9	6.5
VA102	Pioneer	10	7.0
PA878	"	11	2.0
PA875	"	12	7.0
VA103	"	13	2.0

* Represents Pioneer proprietary inbreds; all others are public lines.

** Grey Leaf Spot resistance is scored at Pioneer on a scale of 1 to 9 with 9 being most resistant to the disease. These scores are based on field tests conducted by Pioneer Hi-Bred International, Inc.

1 and 10 mM stock solutions were prepared in DMSO. For stock solutions the amount of cercosporin was calculated from spectrophometric data obtained from a lambda 3 UV/VIS spectrophotometer (Perkin-Elmer) with recorder set at 473 nm using a molar absorbtivity (lambda = 473) of 23,600 (33).

Dark CO_2 fixation assay. The first true leaf of a 3-6 week old plant was obtained from one of the inbreds and trimmed into a strip one centimeter in width and ~20 cm in length. The strip of leaf was then run through a leaf slicer which generated approximately 1 mm X 1 cm leaf slices by means of a rotating, double-edged razor blade (built by the Department of Chemistry, Cornell University, Ithaca NY). The slices were caught in a solution of calcium carbonate:calcium sulfate (1:1), rinsed in deionized distilled water, and blotted dry. (All water referred to as deionized distilled water is 10 mega ohm-cm.) Fifteen slices per vial were placed into 7 ml scintillation vials containing 0.475 ml of 20 mM MOPS buffer at pH 7.0. A total of 1 ul of DMSO or cercosporin from stock solutions was added to the vials. The vials were capped with screw caps fitted with rubber septa and placed into a vacuum desiccator where the DMSO or cercosporin was vacuum infiltrated into the leaf slices for 25 minutes.

After vacuum infiltration, the vials were put into a 30° C circulating water bath with transparent bottom, and the leaf slices were incubated for 60 minutes in continual light (290-330 $uEm^{-2}sec^{-1}$). Light was supplied by 9, 25 watt flood lamps positioned approximately three centimeters beneath the water bath. At the end of the incubation period the vials were covered with aluminum foil sleeves to block out the light. After five minutes in the dark 20 ul of 60 mM sodium bicarbonate containing ^{14}C-labelled sodium bicarbonate

vial through the rubber septum. The slices were allowed to fix CO_2 for 15 minutes and then the reaction was quenched with 0.25 ml TCA:water (1:2 w/v). Excess label was driven off by a stream of air after which 5 ml of 3a70 complete counting cocktail (RPI) was added to each vial (12).

Following the addition of scintillation fluid, the vials were counted in a 1217 rackbeta liquid scintillation counter (LKB Wallac) which calculated the disintegrations per minute (DPM) for each vial. The quench curve used to calculate the DPM/vial was created from a series of vials containing 100 ul of ^{14}C-toulene (4.3×10^4 DPM) (New England Nuclear) and quenched with varying amounts of carbon tetrachloride. The vials also contained 5 ml of scintillation fluid.

Leaf spot assay. Small puncture wounds were made on intact leaves to which deionized distilled water, DMSO, or cercosporin were added. All of the wounds were then covered with a transparent hydrophobic tape (3M) and monitored over a period of a week for the development of lesions. The plants were in the greenhouse under normal growing conditions.

Ion leakage assay. Leaf tissue from the base of the last fully opened leaf was acquired from plants at least 12-15 weeks old but had not flowered. Older tissue was used in this assay since this is the stage of development when the plant is normally infected by Cercospora zeae-maydis (21). This tissue was processed through the leaf slicer as already described. The slices for this assay were 3-4 mm X 1 cm in size. The slices were rinsed in deionized distilled water, blotted dry and placed five to a vial in 7 ml scintillation vials containing 2.49 ml of deionized distilled water. Once the leaf slices were in the vials DMSO or

of 2.5 ml. Aluminum foil sleeves were placed around each vial and then the leaf slices were vacuum infiltrated for 25 minutes.

Following infiltration of DMSO or cercosporin into the leaf slices the vials were placed into a circulating water bath at 25° C as described previously. At this point the conductivity of each vial was measured using a draw-up-electrode on a model 32 conductance meter (Yellow Springs Inc.). The conductivity of each vial was measured over a period of time at prescribed intervals. After fifteen minutes in the water bath the aluminum foil sleeves were removed and the vials exposed from below to continual light, 290-330 $uEm^{-2}sec^{-1}$, for the rest of the assay (10).

After several of the assays were run the method was modified by placing on the vials caps which were fitted with a rubber septum pierced by a stainless steel 3 1/2" 17 gauge needle and a disposable 1" 22 gauge needle. The DMSO or cercosporin was added to the vials via the larger needle by drawing air up and down through the smaller vent needle with a 1 ml syringe. The draw-up electrode was fitted with teflon tubing which mated with the Luer lock on the larger needle in order to bring the solution up into the electrode. Also, the volume of the solution in the vials was increased to 3 ml. These modifications helped to increase the reproducibility of the assay.

For one set of assays another modification was used. The draw-up electrode was replaced by an immersible electrode which gives a continual readout over time. The electrode was immersed in 25 ml of deionized distilled water in a 125 ml erlenmeyer flask. The flask was suspended in the water bath by a clamp attached to a shaker set a 100 rpm. To accomodate the larger

volume fifty leaf slices were used in each flask as well as a 10-fold increase in the volume of DMSO or cercosporin added to each flask.

Statistical analysis of data. The data was analyzed using SAS (SAS Institute Inc.).

RESULTS

Dark CO_2 fixation assay. An initial cercosporin dose response curve was generated for the dark CO_2 fixation assay using concentrations of zero, two, ten, and twenty micromolar cercosporin and leaf material from inbred 7. This assay measures the rate of $^{14}CO_2$ uptake in the dark following a light period. This assay can be used to measure the effects of cercosporin; the more damage the toxin does the lower the CO_2 fixation rate and consequently the lower the ^{14}C uptake. According to the curve a cercosporin concentration of 20 uM gave a significantly lower CO_2 fixation rate than controls (Table 2). A second assay using the same inbred confirmed that 20 uM cercosporin did result in a lower CO_2 fixation rate than did controls. In both trial assays, however, the counts per minute (CPM) for certain vials were too low for the limits of the quench curve. Therefore, the instrument was unable to calculate the disintegrations per minute (DPM), which represent the estimated true amount of radioactivity in the vial.

A new quench curve was generated with a series of quenched ^{14}C standards. The curve utilized an auto window function in the program to calculate DPM from CPM that allows the instrument to adjust the counting window to fit the isotope spectrum's pulse height (Fig. 1).

Table 2. Dark CO_2 fixation assay: cercosporin concentration curve

	Cercosporin Concentration			
	control	2 uM	10 uM	20 uM
DPM	12729.1	8174.2	5354.7	3266.2*

The control value is the mean DPM for 12 repetitions, the other values are the mean DPM for 6 repetitions.

* Some of the DPM values used to calculate this mean were not obtained within the limits of the quench curve used.

The quench curve and the reproducibility of the assay were tested by running a series of assays with inbreds 1 and 9. Each assay was performed with five toxin concentrations: 0, 2, 10, 20, and 100 uM. Based on the data obtained (Table 3) a number of inbreds were assayed at 0, 20, and 100 uM toxin. A total of seven inbreds were tested (Table 4 and Fig. 2). The individual data points used to calculate the means in Table 3 showed considerable variation within an experiment as represented by high coefficients of variation.

Brad Quench 3 ->

01A program mode	1 ->	
01B count mode	4 ->	
02 listing	N ->	
03 time	120 ->	
04 counts 1	900000 ->	
06A auto window	Y ->	14C
06B batch window	N ->	
12 external std time	60 ->	
13 external std counts	900000 ->	
14 print	1,2,5,7,21,22,11,8,10 ->	

Figure 1. Quench curve program, used by the scintillation counter to calculate DPM from CPM.

In addition, only low levels of inhibition of CO_2 fixation (30-40%) were observed even at high cercosporin concentrations. Therefore, a more sensitive method of measuring an inbred's toxin resistance was needed.

Leaf spot assay. The leaf spot assay gave ambiguous results which were hard to quantitate. Lesions of approximately the same size developed on all five inbreds tested: 1, 3, 8, 9, 12 (not shown). Also, lesions similar in appearance to cercosporin-induced lesions developed from some of the DMSO controls. Further development of this assay was not pursued.

Ion leakage assay: toxin concentration curves. Cercosporin, via lipid peroxidation, destroys the integrity of a cell's membrane allowing ions from within the cell to leak out into the surrounding solution. The presence of these ions in solution increases the conductivity of thesolution. Therefore, an increase in conductivity can be used as a measure of

the greater the cellular damage. Using inbred 7, two trial assays were performed. The first assay tested the procedure using a toxin concentration of 5 uM (Table 5). After the initial assay a cercosporin dose response curve was generated using concentrations of 0, 5, 10, and 20 uM cercosporin (Fig. 3).

Table 3. Dark CO_2 fixation assay: results of the first three trials

Inbred	Cercosporin				
	Control	2 uM	10 uM	20 uM	100 uM
1	24973.8*	21528.9	15842.0	13237.3	11691.1
	(22.7)	(36.9)	(42.2)	(44.5)	(47.7)
9	25578.8	24524.4	18034.3	15651.1	16500.7
	(17.7)	(31.4)	(28.3)	(21.1)	(22.2)

* Numbers represent mean DPM. Control values represent 18 repetitions from three trials; all other values represent 9 repetitions from three trials. Numbers in parentheses are coefficients of variance for each mean.

Table 4. Dark CO_2 fixation assay: summary of inbred screening for resistance to cercosporin

Inbred	Cercosporin Concentration		
	Control	20uM	100uM
2	33252.6*	26027.0	26225.5
	(17.0)	(17.5)	(22.5)
3	35470.8	23719.3	27207.1
	(14.4)	(3.7)	(2.9)
5	41430.8	35678.5	29070.6
	(19.6)	(17.3)	(12.5)
7	31733.7	25315.8	21030.9
	(19.6)	(20.3)	(28.7)
8	27425.6	21969.4	19630.4
	(14.1)	(15.9)	(19.6)
9	19475.6	20262.6	13201.5
	(15.3)	(1.4)	(7.5)
12	24459.3	24404.1	20527.0
	(11.5)	(11.0)	(4.5)

* Numbers represent mean DPM. Inbred seven was assayed twice. Control values were derived from 6 repetitions and the other values from 3 repetitions. Numbers in parentheses are coefficients of variance for each mean.

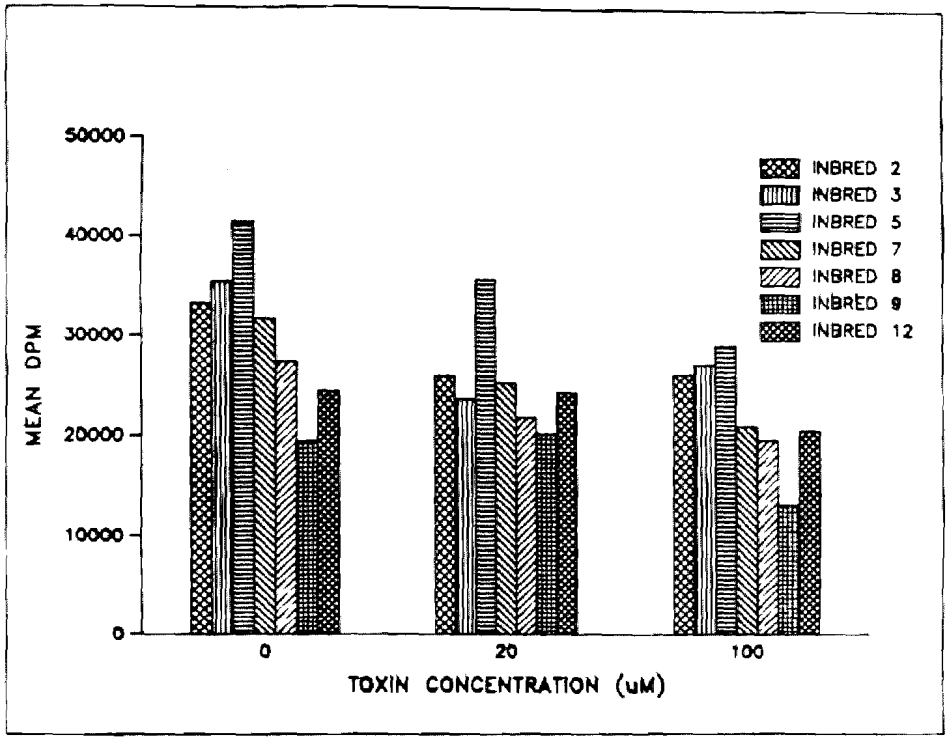


Figure 2. Graph of mean DPM from Table 3.

Table 5. Initial ion leakage assay

Time	Control vials			Experimental vials		
(mins)	1	2	3	4	5	6
5	11.04*	10.38	9.30	10.40	9.74	12.32
10	11.36	10.86	9.87	10.81	10.12	13.08
15	11.88	11.49	10.59	11.50	10.53	13.62
20	12.08	11.90	11.00	11.97	11.05	14.06
25	12.27	12.19	11.50	12.42	11.67	14.41
30	12.48	12.49	11.92	13.00	12.49	14.72
35	12.55	12.90	12.35	13.32	13.01	15.05
40	12.62	13.06	12.84	13.99	13.84	15.51
45	12.62	13.18	13.04	14.76	14.42	16.13
50	12.55	13.58	13.22	15.81	15.53	16.83
55	12.58	13.79	13.58	16.89	16.68	17.84
60	12.55	13.85	13.62	17.78	17.39	18.71

* Conductivity readings for individual vials in initial ion leakage assay. Control vials contained 10 ul DMSO, experimental vials contained 8.75 ul DMSO and 1.25 ul of 10 mM cercosporin in DMSO for a final cercosporin concentration of 5 uM and a final DMSO concentration of 0.4%.

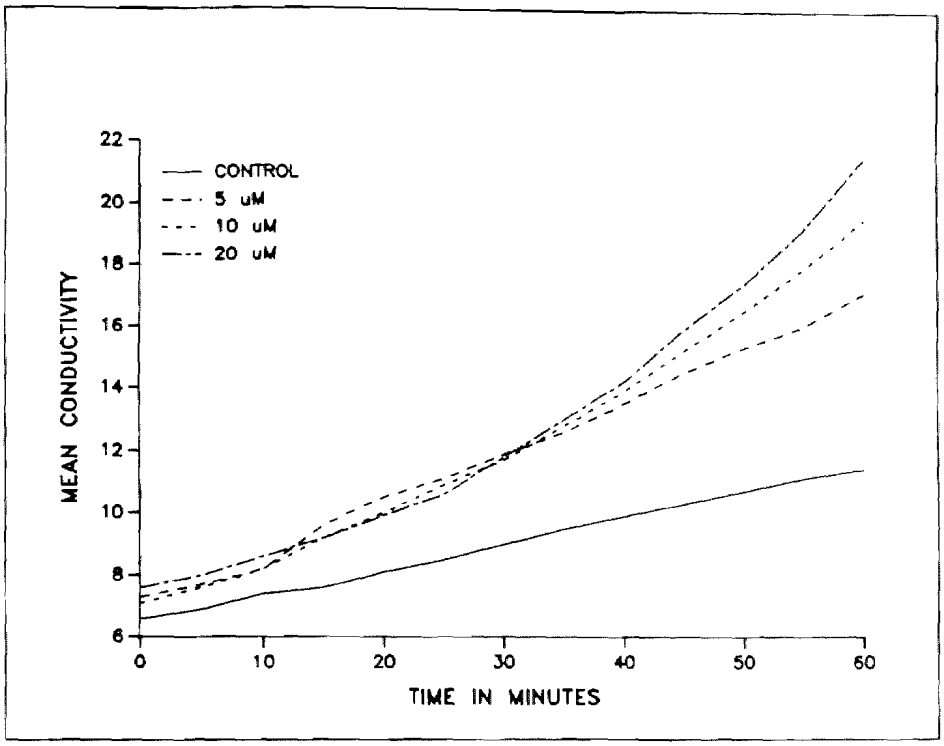


Figure 3. Initial concentration curve for ion leakage assay. Controls contained 10 ul of DMSO plus cercosporin. All other treatments contained an equal volume of DMSO. Inbred 7 was used.

Table 6. Ion leakage assay: Mean conductivity readings ($uEm^{-2}sec^{-1}$) for the cercosporin concentration curves

Inbred/ Toxin conc.	Time 0	15	30	45	60	90	120	150
1 0	9.88*	10.52	11.21	11.65	12.00	12.51	12.96	13.31
0.5	10.05	10.57	11.25	11.70	12.06	12.59	13.05	13.55
1	10.78	11.35	11.87	13.01	13.04	13.67	14.41	15.16
5	9.49	10.04	10.70	11.33	12.16	14.52	17.67	19.79
10	11.00	11.51	12.30	13.18	14.81	19.32	23.40	25.63
5 0	7.29	7.75	8.35	8.79	9.04	9.72	10.38	10.97
0.5	6.72	7.09	7.69	8.13	8.54	8.47	9.85	10.42
1	5.82	6.18	6.75	7.10	7.49	8.08	8.74	9.54
5	5.85	6.26	6.72	7.17	8.06	10.67	13.74	16.63
10	5.63	6.06	6.68	7.57	8.92	13.04	16.99	20.61
7 0	5.84	6.37	7.06	7.53	7.94	8.59	9.06	9.39
0.5	6.04	6.54	7.16	7.65	8.07	8.89	9.48	10.12
1	5.86	6.37	6.97	7.49	7.89	8.73	9.74	11.05
5	7.97	8.47	9.11	10.01	11.52	16.48	20.19	22.58
10	7.74	8.31	9.09	10.59	13.15	19.66	23.26	25.68
9 0	6.67	7.35	8.22	9.01	9.66	10.80	11.50	12.12
0.5	7.92	8.78	9.81	10.60	11.32	12.47	13.39	14.37
1	6.92	7.54	8.35	9.10	9.89	11.03	12.31	13.62
5	8.26	8.91	9.84	11.07	14.22	22.02	27.20	30.36
10	9.39	10.24	11.32	13.21	17.91	27.00	31.22	34.76

* These values represent the mean conductivity readings for the four inbreds tested at zero, 0.5, 1, 5, and 10 uM cercosporin.

Table 7. Slopes of the cercosporin concentration curves

Treatment	Inbred			
	1	5	7	9
control	1.95*	2.29	2.16	3.49
0.5 uM	2.07	2.31	2.59	4.00
1 uM	2.70	2.36	3.31	4.42
5 uM	7.52	7.94	11.43	17.63
10 uM	11.38	11.32	14.25	20.14

*Slopes are from the best fitting lines generated from data in Table 9 using a linear regression analysis (see text).

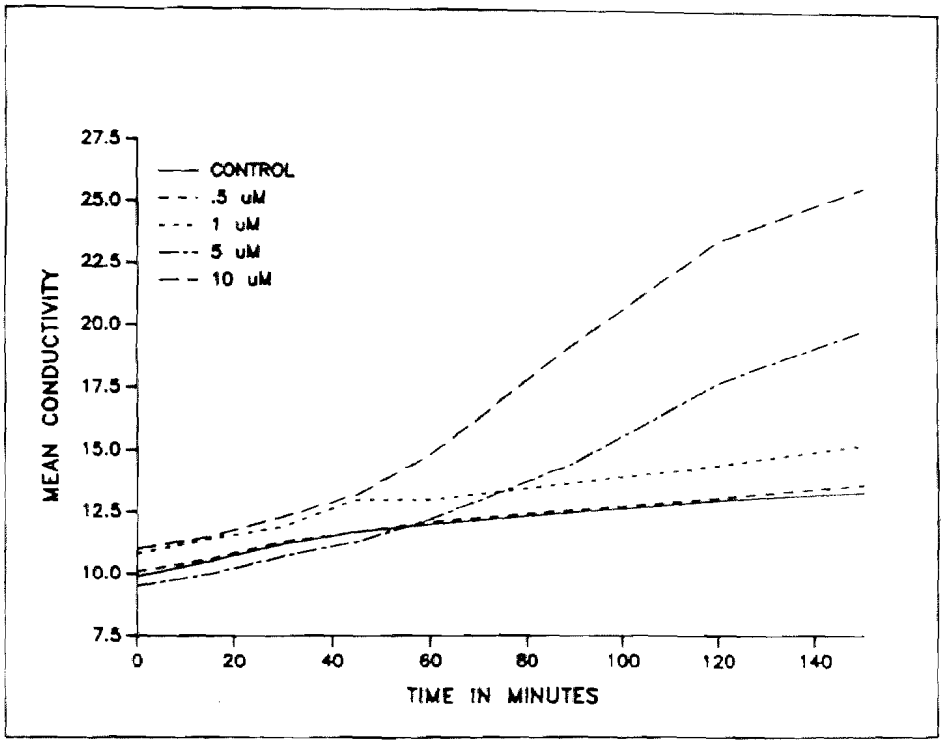


Figure 4. Graph of the concentration curves for zero, 0.5, 1, 5, and 10 uM cercosporin. Controls contained 10 ul DMSO. All other treatments contained an equal volume of DMSO. The curves were created from data found in Table 6. Slopes of the lines can be found in Table 7. Figures 5, 6, and 7 represent same information for inbreds 5, 7, and 9.

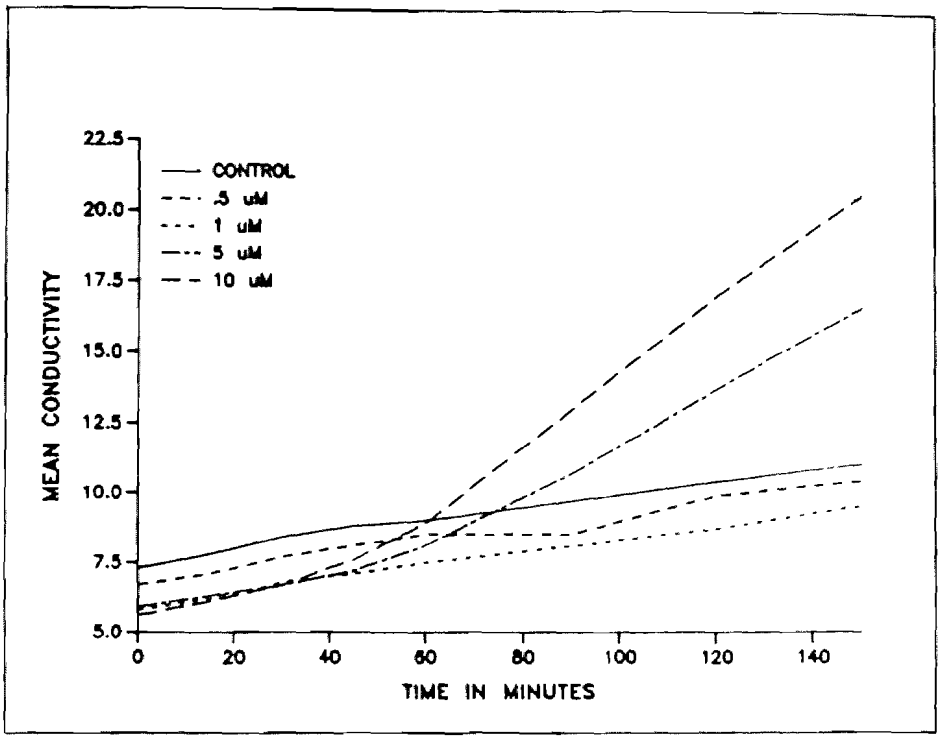
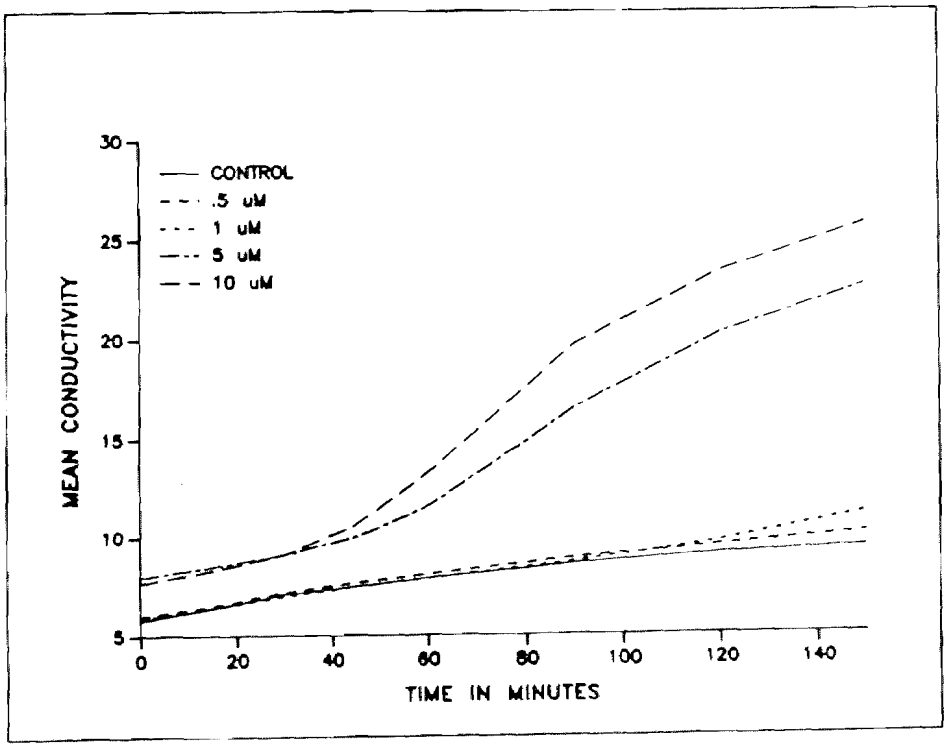


Figure 5. Ion Leakage Assay: Concentration Curve, Inbred number five



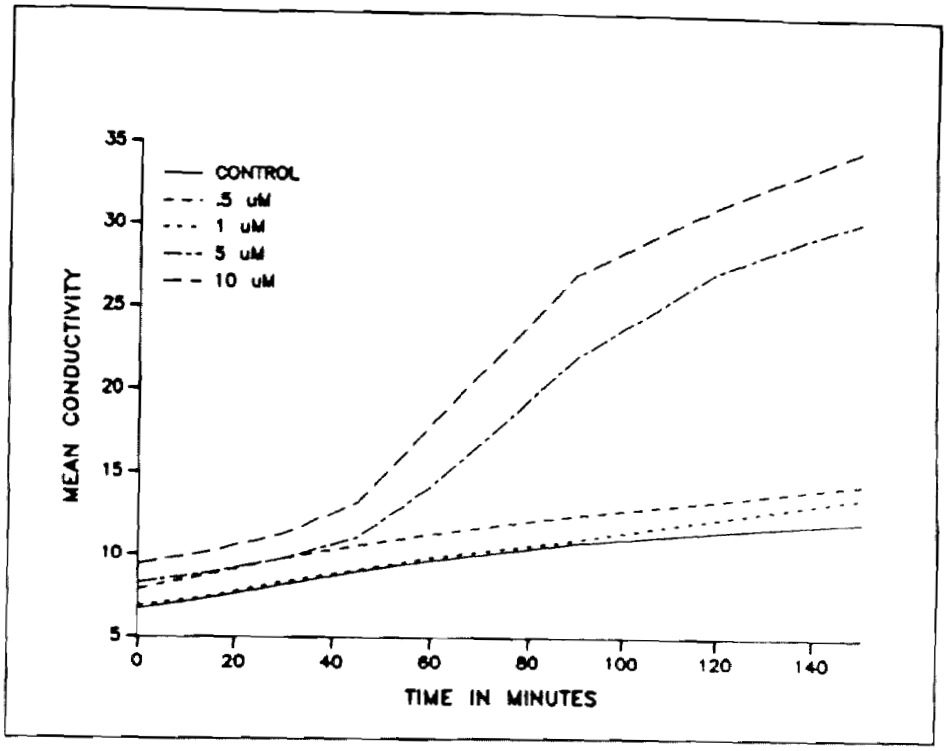


Figure 7. Ion Leakage Assay: Concentration Curve, Inbred number nine

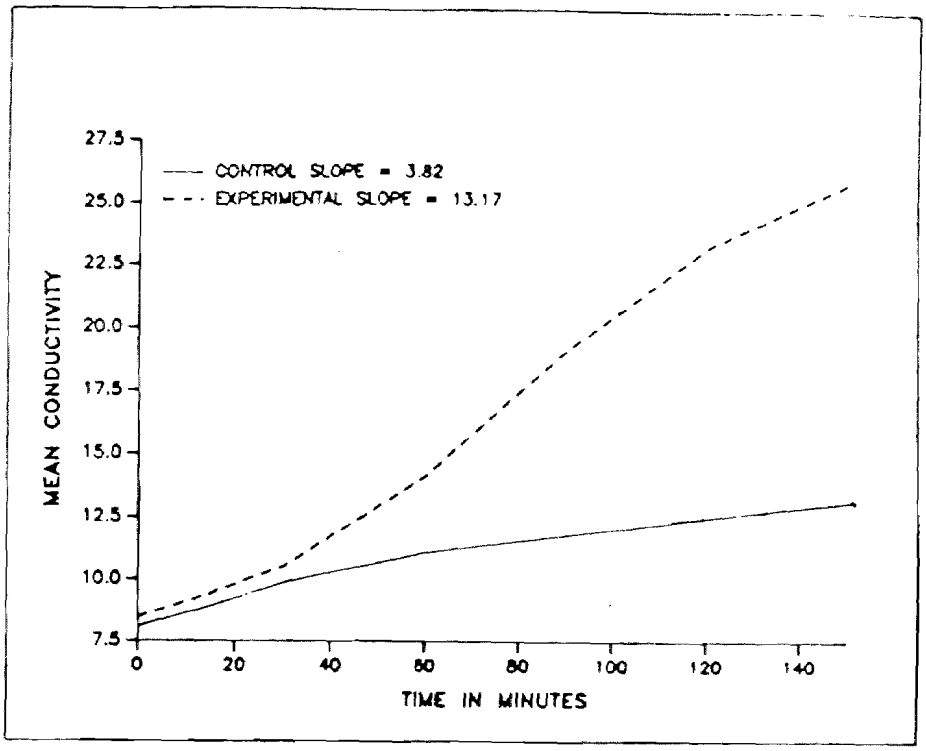


Figure 8. Curve of mean conductivities over time for inbred 1. Data points for curve can be found in Table 8. Slopes of lines were generated by a linear regression analysis (as described in text the slopes have been multiplied by 100). Controls contained 10 ul DMSO. Experimental vials contained 7.0 ul DMSO and 3.0 ul of 10 mM cercosporin in DMSO for a final toxin concentration of 10 uM. Figures 9-20 represent same curves generated for inbreds 2-13 respectively.

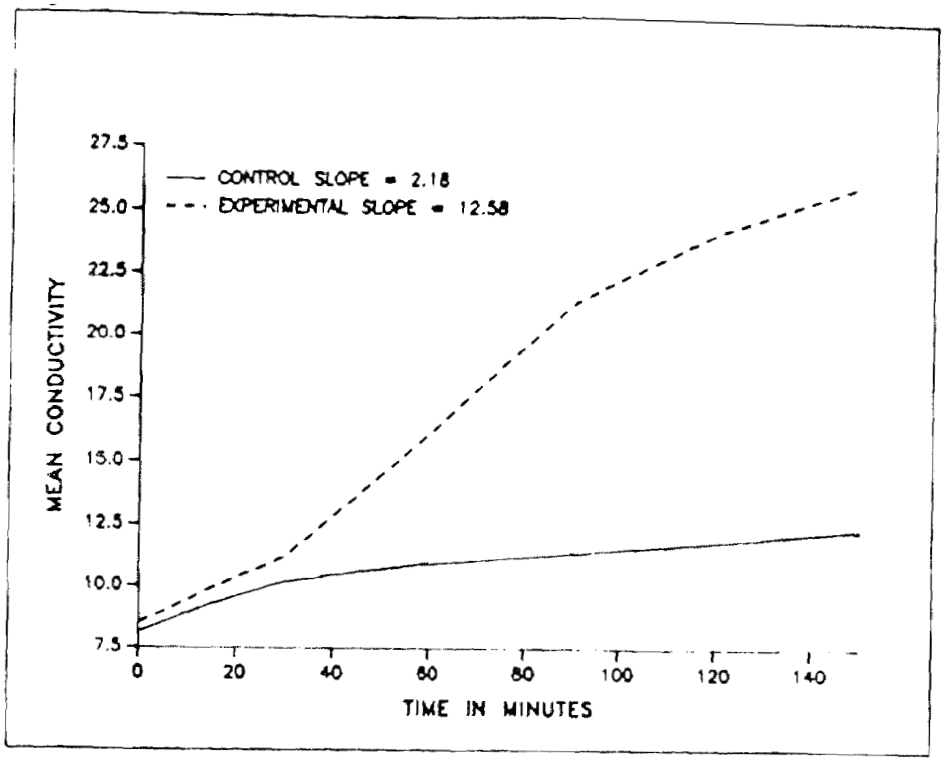


Figure 9. Ion Leakage Assay: Inbred #2 Summary

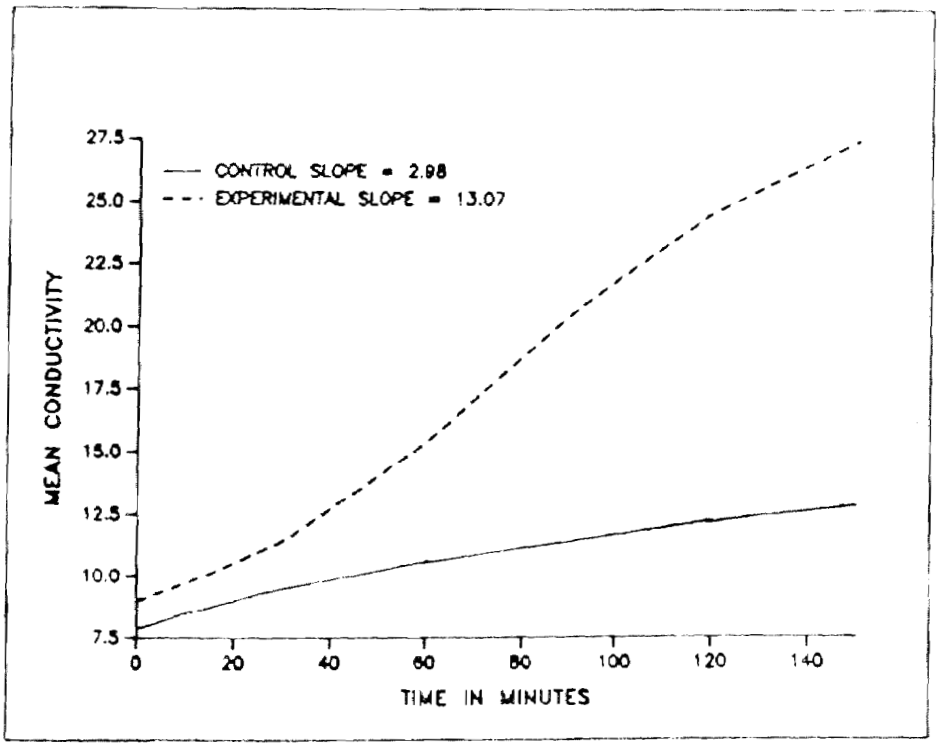


Figure 10. Ion Leakage Assay: Inbred #3 Summary

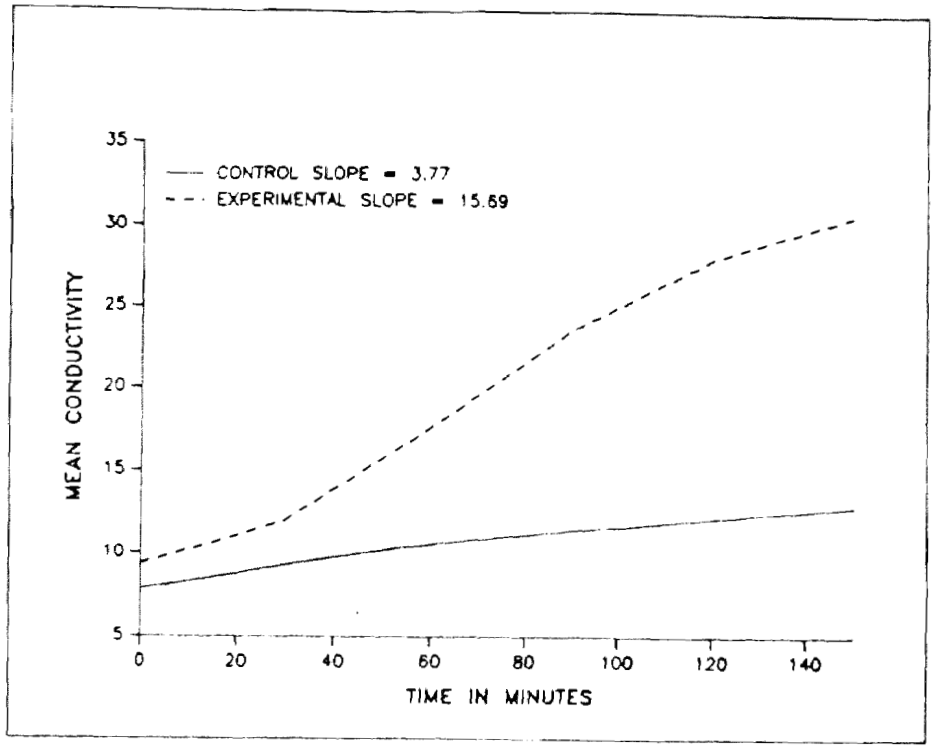
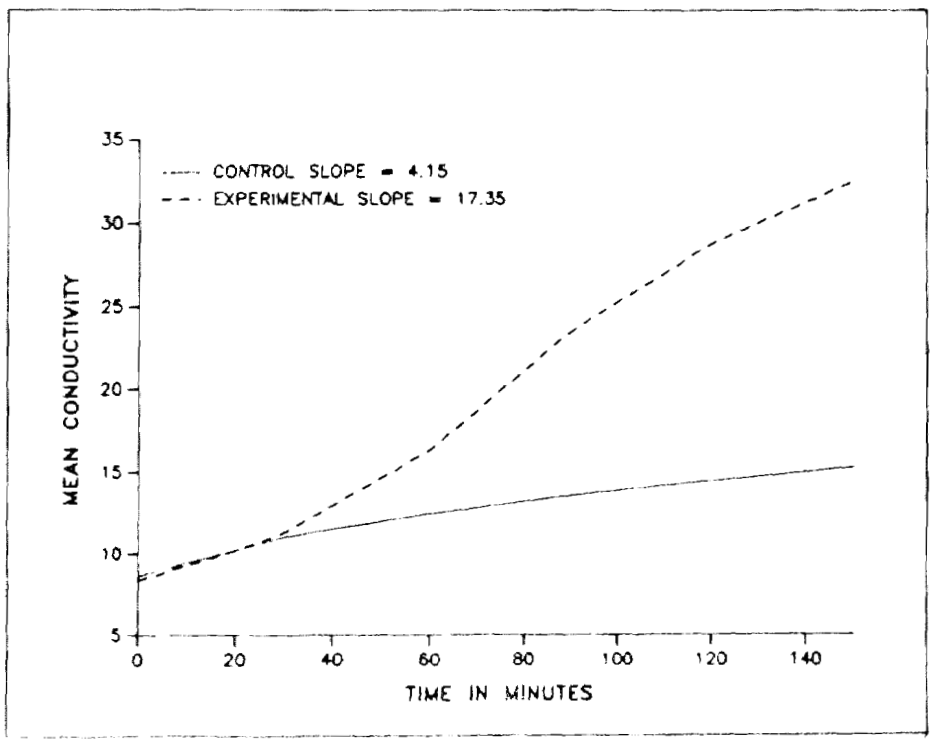


Figure 11. Ion Leakage Assay: Inbred #4 Summary



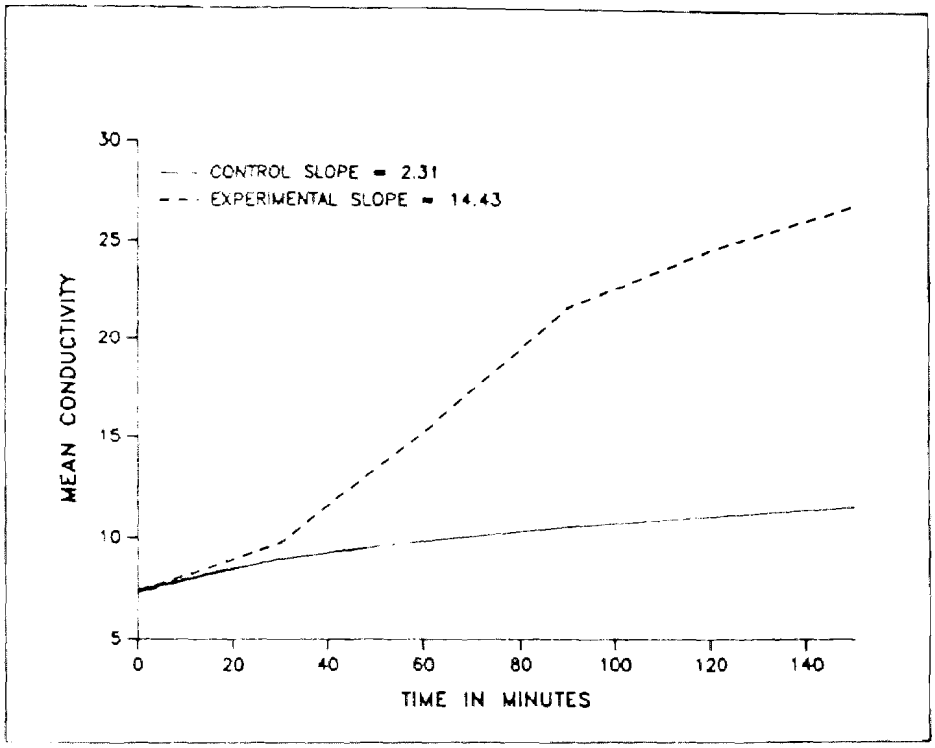
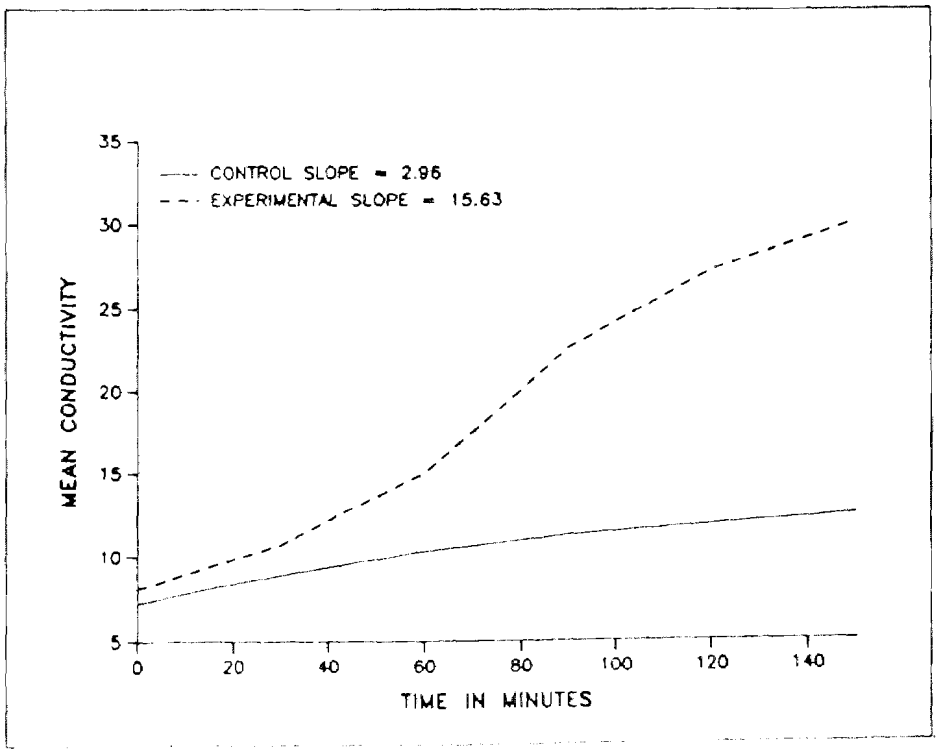


Figure 13. Ion Leakage Assay: Inbred #6 Summary



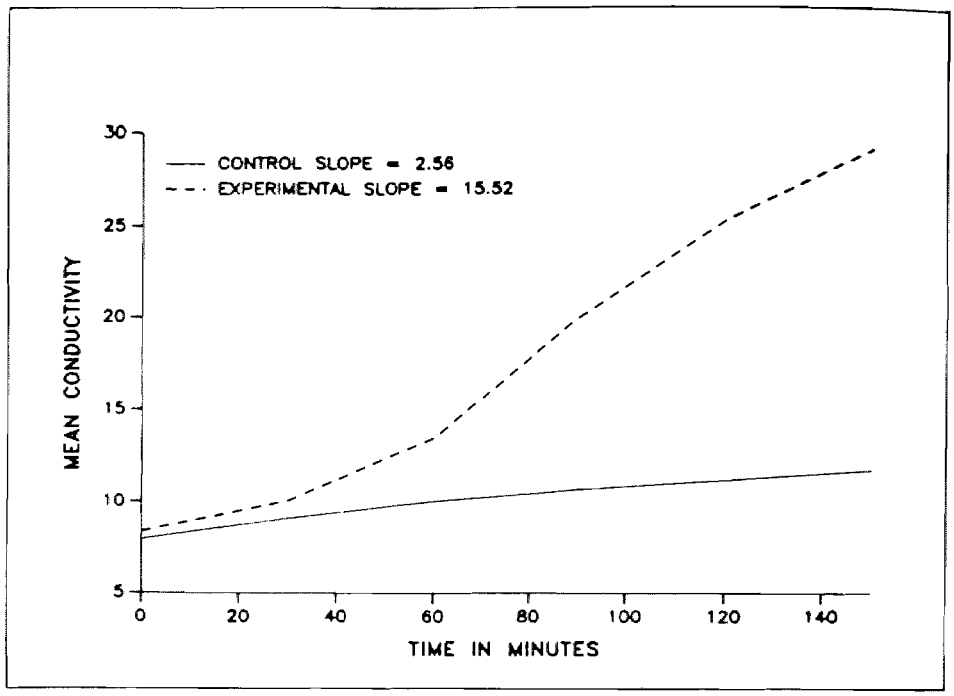


Figure 15. Ion Leakage Assay: Inbred #8 Summary

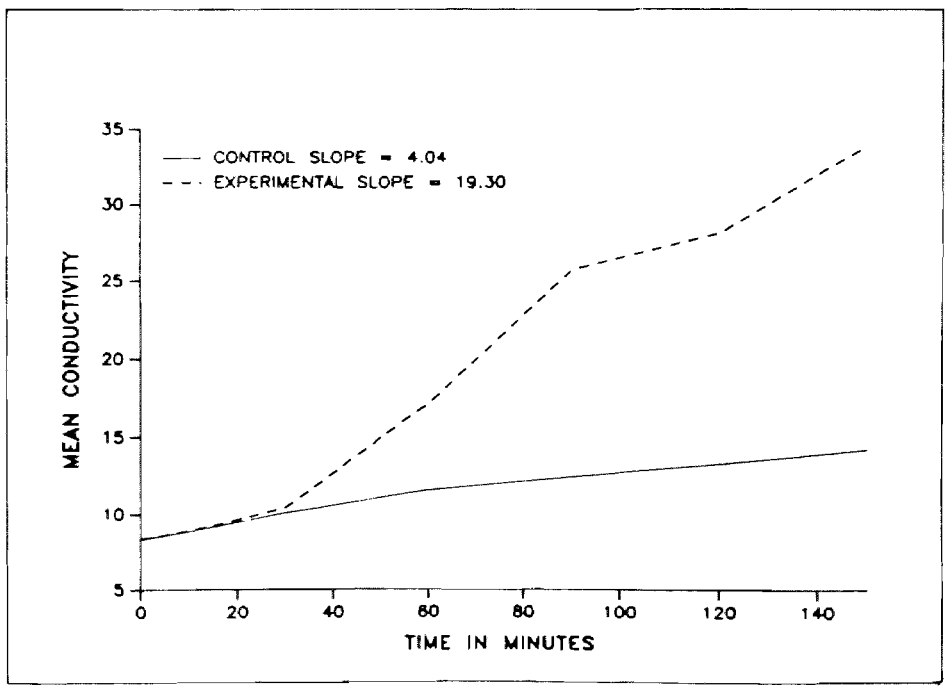


Figure 16. Ion Leakage Assay: Inbred #9 Summary

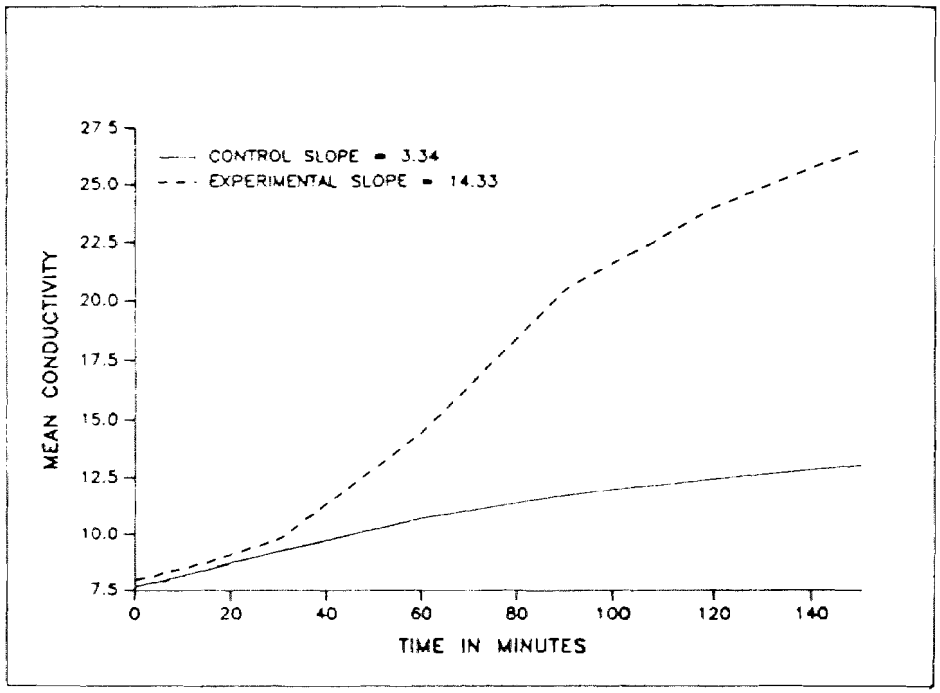


Figure 17. Ion Leakage Assay: Inbred #10 Summary

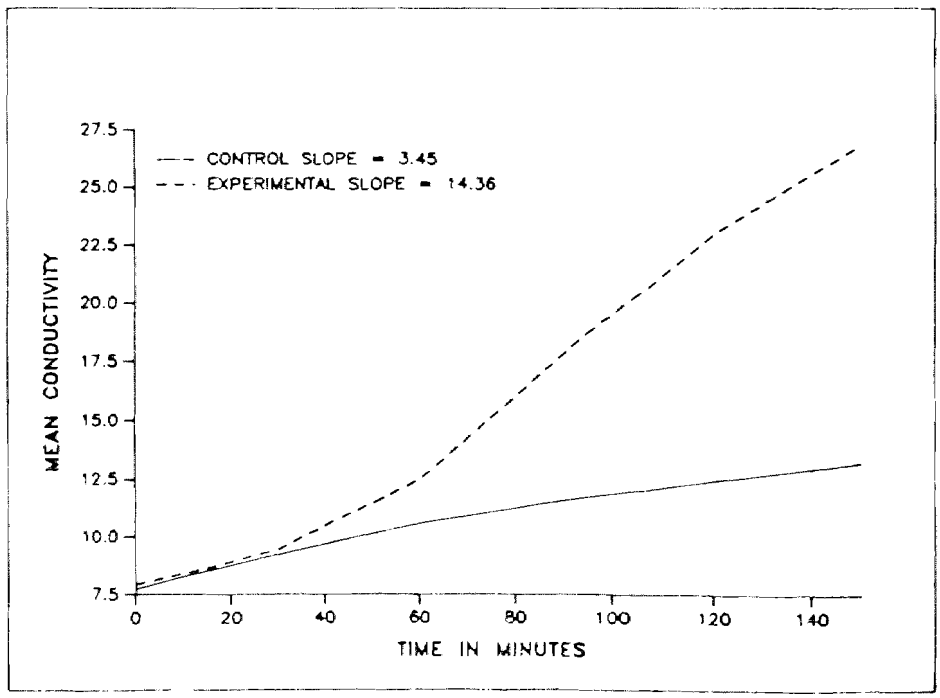


Figure 18. Ion Leakage Assay: Inbred #11 Summary

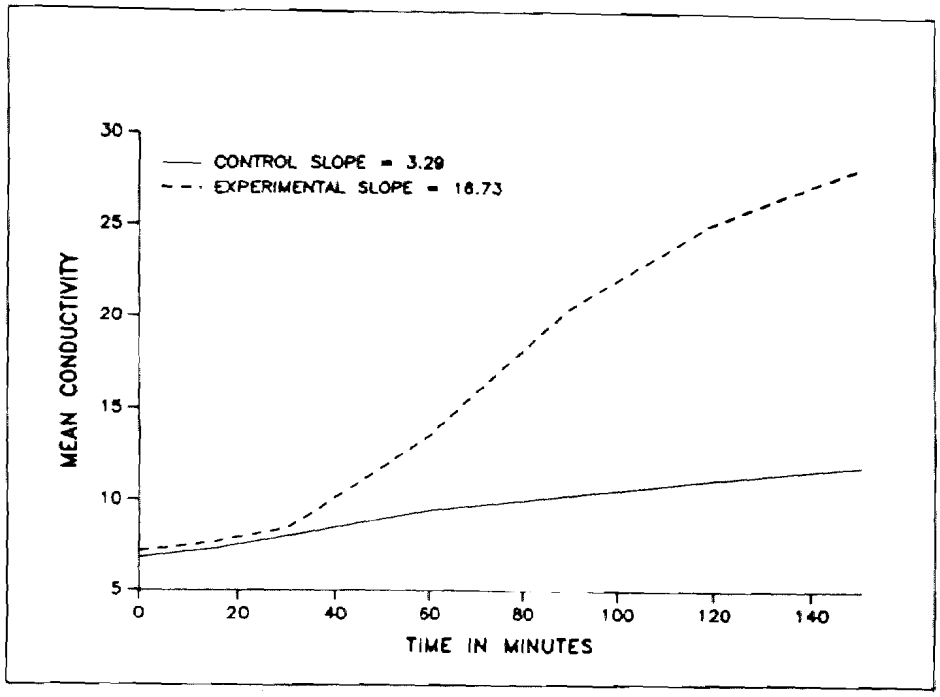


Figure 19. Ion Leakage Assay: Inbred #12 Summary

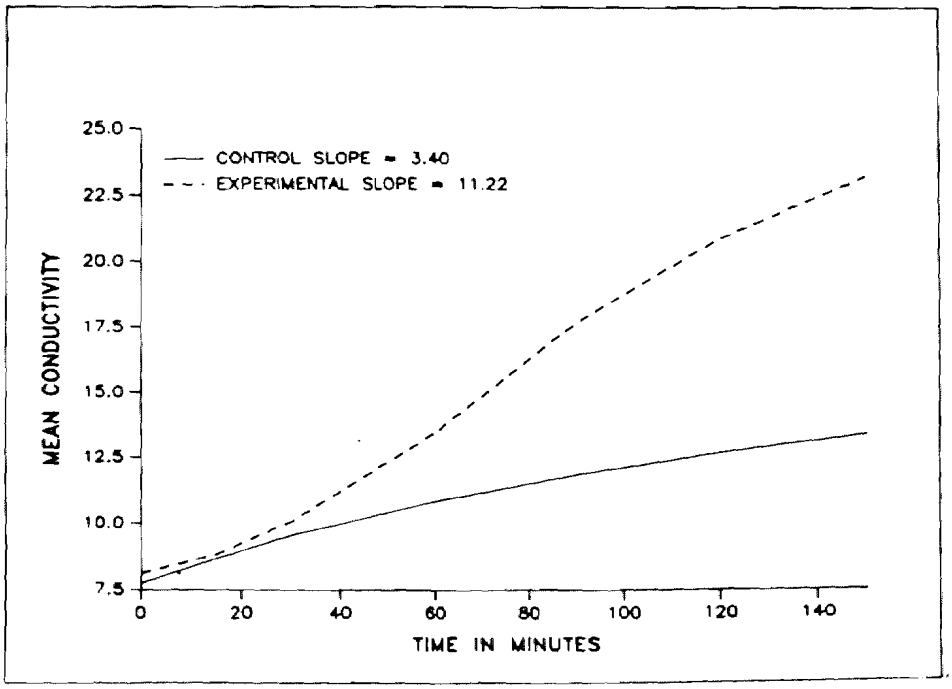


Figure 20. Ion Leakage Assay: Inbred #13 Summary

These values were then analyzed using the general linear models function in the SAS statistics package. The regression determines a line of best fit for the entire curve from which the slope is calculated. The slopes of the lines derived from the experimental treatments (those vials containing cercosporin) differed significantly from the slopes of the lines derived from the control treatments (Table 9).

In addition, the mean increase in conductivity of the experimental vials versus the control vials was calculated using Equation 1 (Table 9).

Equation 1.

$[(P - Q) - (S - T)] / n$

P = the conductivity of the experimental vials at time = X

Q = the mean conductivity of the control vials at time = X

S = the mean conductivity of the experimental vials at time = 0

T = the mean conductivity of the control vials at time = 0

n = the number of experimental vials

Table 8. Ion leakage assay: Mean conductivities ($uEm^{-2}sec^{-1}$)

Inbred	Time in Minutes						
	zero	15	30	60	90	120	150
1	8.09*	8.93	9.89	11.12	11.82	12.52	13.16
	8.47	**9.44**	**10.54**	**14.12**	**19.10**	**23.21**	**25.83**
2	8.10	9.28	10.19	10.94	11.35	11.86	12.27
	8.48	**9.92**	**11.19**	**16.12**	**21.36**	**24.24**	**26.15**
3	7.87	8.73	9.51	10.57	11.40	12.18	12.87
	9.00	**10.10**	**11.39**	**15.38**	**20.46**	**24.57**	**27.45**
4	7.86	8.56	9.33	10.60	11.43	12.15	12.87
	9.35	**10.62**	**11.98**	**17.62**	**23.57**	**28.02**	**30.61**
5	8.62	9.81	10.99	12.46	13.54	14.42	15.25
	8.33	**9.68**	**11.22**	**16.34**	**23.41**	**28.75**	**32.46**
6	7.29	8.19	8.95	9.86	10.58	11.08	11.54
	7.41	**8.53**	**9.74**	**15.19**	**21.62**	**24.61**	**26.83**
7	7.27	8.17	8.92	10.33	11.27	11.86	12.50
	8.13	**9.40**	**10.72**	**14.97**	**22.58**	**27.25**	**30.07**
8	7.94	8.52	9.07	10.01	10.65	11.14	11.70
	8.36	**9.17**	**10.01**	**13.45**	**20.00**	**25.35**	**29.14**
9	8.40	9.15	10.09	11.58	12.48	13.32	14.21
	8.38	**9.22**	**10.41**	**17.12**	**25.73**	**28.16**	**33.84**
10	7.68	8.41	9.27	10.74	11.74	12.42	13.01
	7.95	**8.72**	**9.81**	**14.45**	**20.44**	**23.95**	**26.49**
11	7.75	8.51	9.25	10.60	11.59	12.45	13.22
	7.93	**8.62**	**9.47**	**12.48**	**17.79**	**22.95**	**26.82**
12	6.83	7.33	8.03	9.43	10.25	11.13	11.84
	7.17	**7.69**	**8.44**	**13.46**	**20.45**	**25.14**	**28.24**
13	7.75	8.68	9.56	10.84	11.84	12.68	13.37
	8.12	**8.83**	**10.07**	**13.41**	**17.61**	**20.85**	**23.18**

* Values represent mean conductivity readings. Inbreds 2-8,10,11, and 13 were assayed three times. Inbreds 1 and 9 were assay four times and inbred 12 only twice. Upper values = control values, and lower boldface values = experimental values.

Table 9. Ion leakage assay: Slopes of control and experimental lines and mean increases in conductivity at T = 150.

Inbred	GLS Score*	Slope exp.**	Slope con.**	Mean conductivity increase T=150***
1	2.0	13.17	3.82	12.28
2	7.3	12.58	2.18	13.51
3	2.5	13.07	2.98	13.45
4	6.0	15.69	3.77	16.25
5	7.0	17.35	4.15	17.51
6	3.0	14.43	2.31	15.17
7	3.0	15.63	2.96	16.70
8	6.5	15.52	2.56	17.03
9	6.5	19.30	4.04	19.65
10	7.0	14.33	3.34	13.20
11	2.0	14.36	3.45	13.42
12	7.0	16.73	3.29	16.07
13	2.0	11.22	3.40	9.45

* Grey Leaf Spot scores are based on a scale from 1 to 9 with 9 being the most resistant.

** Slopes represent slopes of best fitting line determined by a linear regression analysis (see text).

*** Mean conductivity increase was figured using equation 1.

The mean increase in conductivity was calculated for the data points at time = 150 and time = 15 minutes. This data was then analyzed by performing a Duncan's multiple range test to see if the increase in conductivity of one inbred differed significantly from another. At time = 15 conductivity values for all the inbreds were not significantly different from each other (Table 10), but at time = 150 significant differences between inbreds were apparent (Table 11). A Duncan's analysis was not done for the slopes of the lines.

Correlation coefficients were calculated for both experimental and control slopes, mean increases in conductivity at T = 150, and Gray Leaf Spot (GLS) scores (Table 12). As would be expected, a strong correlation (0.9367) was found between the mean increases in conductivity at T = 150 and the slopes of the experimental lines. The experimental slopes and the mean increases in conductivity at time = 150 both showed a somewhat weak but positive correlation to the inbreds' Grey Leaf Spot scores. The correlation values were 0.5399 and 0.5524 respectively. Thus, the more resistant the inbred the more ion leakage occurred, which is the reverse of what was expected according to the original hypothesis.

Table 10. Duncan's test at T = 15 for the mean increase in conductivity of the experimental vials versus the control vials

Inbred	N*	Mean**	GLS score	Duncan grouping***
4	13	0.57	6.0	A
7	13	0.37	3.0	A
2	13	0.26	7.3	A
3	13	0.24	2.5	A
8	12	0.23	6.5	A
6	13	0.22	3.0	A
5	13	0.16	7.0	A
1	16	0.14	2.0	A
9	18	0.09	6.5	A
10	14	0.04	7.0	A
12	9	0.02	7.0	A
11	13	-0.07	2.0	A
13	13	-0.22	2.0	A

* Total number of experimental vials assayed for each inbred.

** Mean increase in conductivity derived from equation 1.

*** Groups with same letter are not significantly different.

Table 11. Duncan's test at T = 150 for the mean increase in conductivity of the experimental vials versus the control vials

Inbred	N*	Mean**	GLS Score	Duncan Grouping***					
9	18	19.65	6.5	A					
5	13	17.51	7.0	A	B				
8	12	17.03	6.5	A	B	C			
7	13	16.70	3.0	A	B	C	D		
4	13	16.25	6.0	A	B	C	D		
12	9	16.07	7.0	A	B	C	D		
6	13	15.17	3.0		B	C	D	E	
2	13	13.51	7.3			C	D	E	
3	13	13.45	2.5			C	D	E	
11	13	13.42	2.0			C	D	E	
10	14	13.20	7.0				D	E	
1	16	12.28	2.0					E	F
13	14	9.45	2.0						F

* Total number of experimental vials assayed for each inbred.

** Mean increase in conductivity derived from equation 1.

*** Groups with same letter are not significantly different.

Table 12. Correlation coefficients for thirteen inbreds screened for cercosporin resistance (Prob > |R| Testing RHO=0)*.

Variable	GLS score	Slope exp.	Slope con.	Mean increase in conductivity at T=150
GLS score	1.0000	0.5399	0.0371	0.5524
Prob > |R|	0.0000	0.0548	0.9040	0.0483
Slope exp.	0.5399	1.0000	0.4423	0.9367
Prob > |R|	0.0548	0.0000	0.1278	0.0000
Slope con.	0.0371	0.4423	1.0000	0.1839
Prob > |R|	0.9040	0.1278	0.0000	0.5466
Mean inc.	0.5524	0.9367	0.1839	1.0000
Prob > |R|	0.0483	0.0000	0.5466	0.0000

* A correlation of 1.0000 means the two variables are perfectly correlated, but as correlation approaches zero the two variables become less strongly correlated. Prob>|R| is the probability that correlation is actually zero when it is assumed that there is a correlation between the two values. Therefore, the smaller the prob>|R| value is the more confident one can be in the correlation.

Not only was the correlation of cercosporin resistance and disease resistance the opposite of what was expected, none of the inbreds tested showed a significantly higher level of resistance to cercosporin above the rest. Based on the slopes of the experimental lines and the mean increases in conductivity at T = 150, only about a two-fold difference in the level of cercosporin susceptibility exists between the most resistant inbred, 13, and the least resistant inbred, 9 (Table 9).

Ion leakage assay: dark controls. For each inbred one trial was run which also included a dark toxin control. These vials were treated the same as the experimental vials except that they were never exposed to light. These vials behaved like the controls without toxin, showing a minimal increase in conductivity over time (Table 13). The similarity of conductivity increase for dark toxin and no toxin controls was confirmed once for each inbred; subsequently, the dark toxin control was not run.

Ion leakage assay: immature tissue. Inbreds 1 and 4 were also assayed using younger leaf tissue than that used in the rest of the assays to determine whether the response to cercosporin would be different from more mature tissue. These inbreds represented a disease resistant line and a disease susceptible line. The tissue, instead of coming from the last fully opened leaf on a 12-15 week old plant, was taken from the first true leaf of a 4-6 week old plant. The slopes of the lines generated from the data for the experimental treatment were 23.40 and 21.83 (Tables 14 and 15) for the more immature tissue of inbreds 1 and 4 respectively as compared to 13.17 and 15.69 (Table 9) for the more mature tissue. Thus immature tissue was less tolerant to cercosporin than mature tissue. This was not investigated further, however, since we were

most interested in the response of mature tissue at the time infection normally occurs.

Ion leakage assay: toxin effects. One experiment performed in a dark room (data not shown) using inbreds six and nine showed higher initial conductivities for vials that contained cercosporin as compared to vials without cercosporin. Over a period of 60 minutes, however, the increase in conductivity of the experimental vials was the same as the controls. Therefore, to be sure that cercosporin itself did not change the conductivity of the solution, several assays were performed with toxin in the absence of tissue. To observe any change in conductivity when cercosporin was added to the solution an immersible electrode replaced the draw-up electrode. Whetherirradiated or non-irradiated at the time of adding cercosporin to the solution a jump of approximately 2 $uEm^{-2}sec^{-1}$ was observed (Figs. 21-24). When tissue was present this jump in conductivity was not observed (Figs. 25, 26). When this experiment was repeated using the draw-up electrode the sharp increase in conductivity was absent even without tissue in the solution (Table 16). Unable to reproduce the results using a different electrode, the effect of toxin on conductivity was not pursued further.

Table 13. Ion leakage assay: Dark toxin controls versus no toxin controls

Inbred	Slope dark con.*,**	Slope con.
1	5.31	3.82
2	3.84	2.18
3	4.17	2.98
4	5.18	3.77
5	5.12	4.15
6	1.72	2.31
7	4.87	2.96
8	3.33	2.56
9	4.00	4.04
10	4.55	3.34
11	4.35	3.45
12	2.53	3.29
13	5.15	3.40

* Slopes represent slopes of best fitting line as determined by a linear regression analysis (see text).

** Dark toxin controls represent only four reps for each inbred.

Table 14. Ion leakage assay: Mean conductivities from testing immature tissue

Inbred	Time in Minutes						
	zero	15	30	60	90	120	150
1							
control	10.56	11.93	13.95	15.43	16.32	17.20	18.31
dark control	11.92	14.16	16.26	18.32	20.39	22.44	23.93
experimental	10.29	11.94	13.91	21.83	30.83	37.87	41.93
4							
control	8.59	9.79	10.77	12.11	13.09	14.07	16.48
dark control	8.96	10.32	11.70	13.40	14.93	16.54	17.93
experimental	9.57	11.30	13.05	19.72	28.60	35.05	39.65

Mean conductivity readings for tissue taken from the first true leaf of a four week old plant. Dark controls were vials containing toxin but were not exposed to light at time = 15 minutes. A cercosporin concentration of 10 uM was used in both experimental vials and dark control vials.

Table 15. Ion leakage assay: Slopes of lines from testing immature tissue

Treatment	Inbred	
	1	4
control line	4.82	4.67
dark control line	7.67	5.77
experimental line	23.40	21.83

Slopes of best fitting lines generated from data in Table 14. Slopes were determined by a regression analysis (see text).

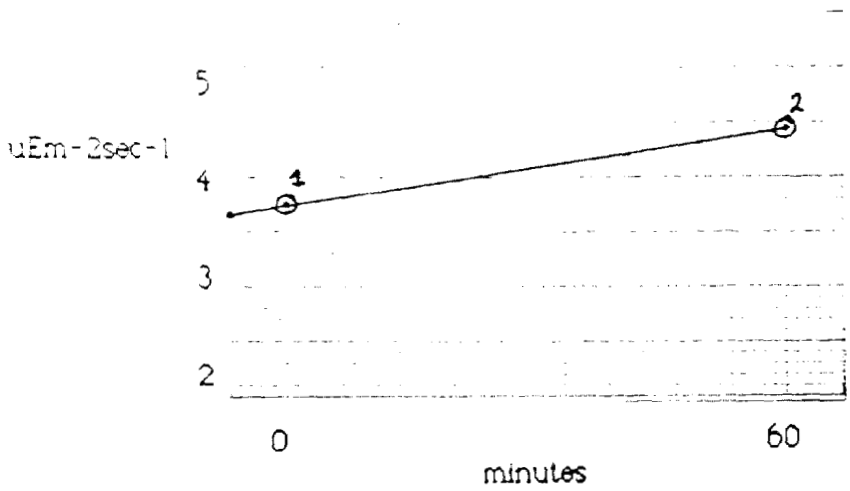


Figure 21. Conductivity of water.
1 = 3.74 $uEm^{-2}sec^{-1}$, 2 = 4.43 $uEm^{-2}sec^{-1}$.

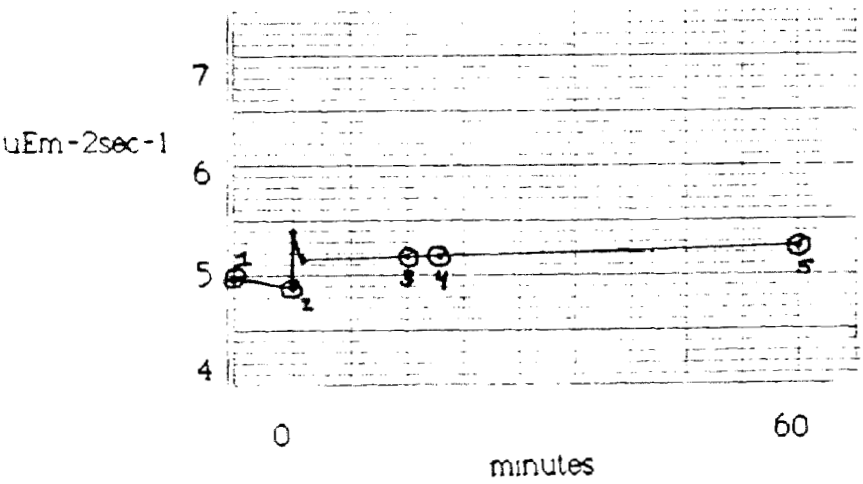


Figure 22. Conductivity of water and DMSO.
1 = 4.97 $uEm^{-2}sec^{-1}$, 2 = DMSO addition, 3 = 5.15 $uEm^{-2}sec^{-1}$, 4 = light exposure, and 5 = 5.22 $uEm^{-2}sec^{-1}$.

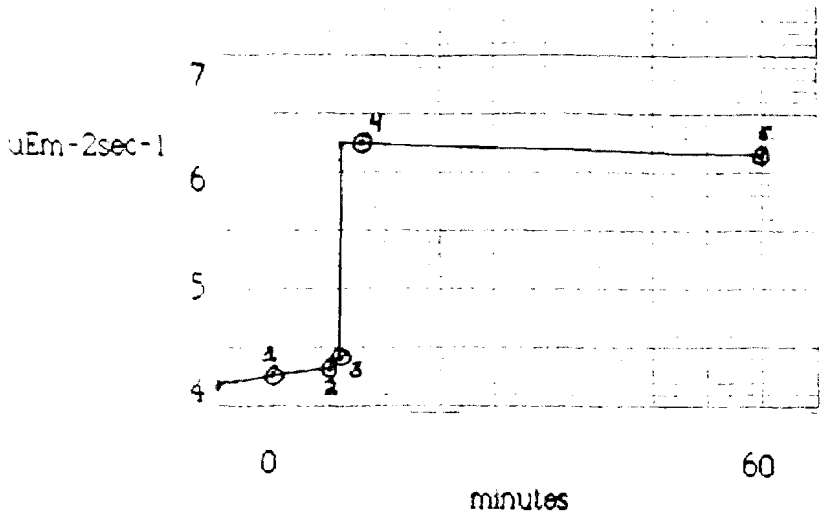


Figure 23. Conductivity of water and cercosporin added in the light. 1 = 4.27 $uEm^{-2}sec^{-1}$, 2 = DMSO addition, 3 = toxin, 4 = 6.23 $uEm^{-2}sec^{-1}$, and 5 = 6.13 $uEm^{-2}sec^{-1}$.

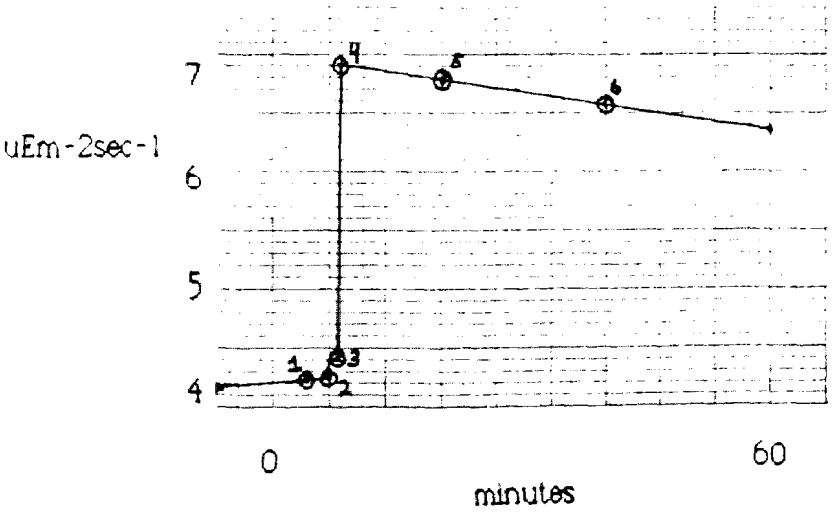


Figure 24. Conductivity of water and cercosporin added in the dark. 1 = 4.15 $uEm^{-2}sec^{-1}$, 2 = DMSO addition, 3 = toxin addition, 4 = 6.90 $uEm^{-2}sec^{-1}$, 5 = light exposure, and 6 = 6.59 $uEm^{-2}sec^{-1}$.

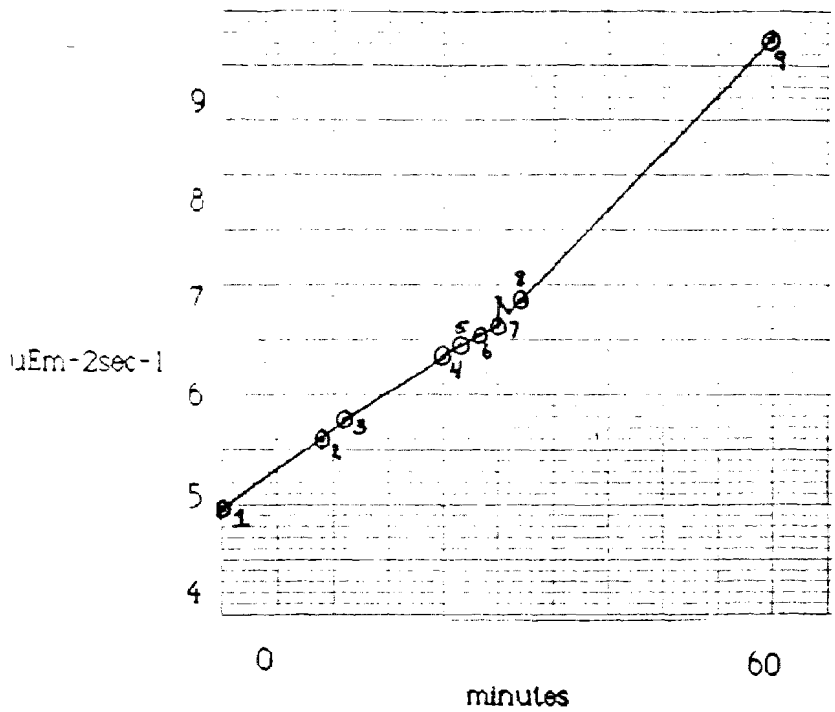


Figure 25. Conductivity of plant tissue and cercosporin added in the light. 1 = 4.97 $uEm^{-2}sec^{-1}$, 2 = light exposure, 3 = an accidental jarring of electrode, 4 = 6.35 $uEm^{-2}sec^{-1}$, 5 = DMSO addition, 6 = 6.63 $uEm^{-2}sec^{-1}$, 7 = toxin addition, 8 = 6.85 $uEm^{-2}sec^{-1}$, and 9 = 9.21 $uEm^{-2}sec^{-1}$.

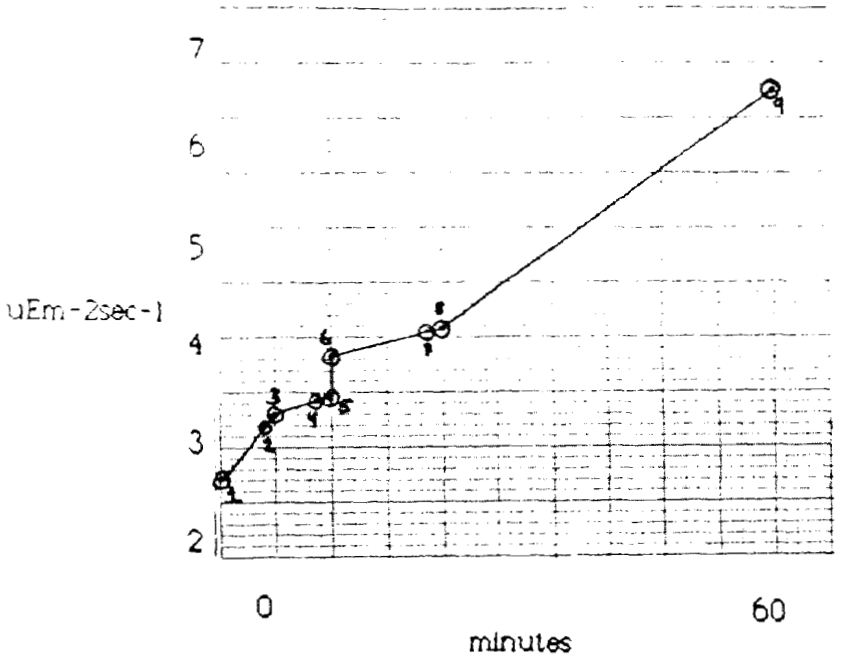


Figure 26. Conductivity of plant tissue and cercosporin added in the dark. 1 = 2.71 $uEm^{-2}sec^{-1}$, 2 = 3.20 $uEm^{-2}sec^{-1}$, 3 = DMSO addition, 4 = 3.48 $uEm^{-2}sec^{-1}$, 5 = toxin addition, 6 = 3.83 $uEm^{-2}sec^{-1}$, 7 = 4.05 $uEm^{-2}sec^{-1}$, 8 = light exposure, and 9 = 6.25 $uEm^{-2}sec^{-1}$.

Table 16. Effects of cercosporin* on conductivity in both dark and light

Treatment/ time	Cercosporin concentration					
	Water control	DMSO control	.5 uM	1 uM	5 uM	10 uM
Light**						
0	3.24	3.76	3.18	3.44	4.55	3.43
10	3.43	3.91	3.48	3.68	4.76	3.94
20	3.60	4.17	3.74	4.14	5.11	4.36
30	3.78	4.34	3.91	4.29	5.25	4.53
40	3.93	4.57	4.10	4.43	5.43	4.72
50	4.12	4.78	4.26	4.57	5.79	4.89
60	4.29	4.97	4.42	4.78	5.97	5.03
Dark***						
0	3.56	3.03	4.49	4.13	4.45	4.67
10	4.05	3.43	4.83	4.78	4.71	5.16
20	4.26	3.86	5.02	4.95	5.03	5.51
30	4.43	4.13	5.22	5.03	5.13	5.65
40	4.59	4.35	5.45	5.16	5.28	5.84
50	4.77	4.59	5.64	5.28	5.41	6.06
60	4.93	4.77	5.90	5.39	5.57	6.23

* Plant tissue was not present in this experiment.

** In light treatment, the vials were exposed to light at fifteen minutes as in a normal ion leakage assay.

*** In dark treatment, vials remained wrapped in an aluminum foil sleeve in order to block out all light. DMSO and cercosporin were added to vials between 10 and 20 minutes readings.

DISCUSSION

The dark CO_2 fixation assay is designed to measure the amount radioactive CO_2 fixed by plant cells during the dark phase of photosynthesis. Following a photo-incubation period plant cells in the dark reduce CO_2 and combine it with hydrogen to form a variety of carbohydrates. If cercosporin is present in the system during the photo-incubation period, it will destroy the cell inhibiting CO_2 fixation. Therefore, damage caused by cercosporin can be detected by looking for decreased levels of radioactivity in the vials which contain toxin as compared to control vials. Too much variation between repetitions of the same treatment, as represented by large coefficients of variance (Table 3,4), in conjunction with large background fixation rates, however, made it difficult to conclude whether differences in the rate of $^{14}CO_2$ fixation among samples were due to cercosporin or to sample variation.

The variation within treatment groups is probably due to leaf slices of differing quality since even the control groups had large coefficients of variance. If the amount of leaf tissue in each repetition was not identical, those vials which contained more material, and consequently a greater number of viable cells, would exhibit higher amounts of $^{14}CO_2$ fixation. Differences in the number of viable cells per vial is probably due to a combination of the following. First, some of the leaf slices in one vial might be slightly larger than those in another vial, and second, the number of cells damaged by the slicing process may not always be equivalent. Tissue age may be another factor leading to variation within treatment groups. Despite the use of only one leaf per experiment and selection of leaf slices at random for

each vial, it is possible that the leaf slices in one vial may have come more from the tip of the leaf than the base. Tissue at the tip of the leaf is younger than tissue from the base and consequently could be metabolically more active allowing it to fix a larger amount of CO_2. Also, other researchers (5) have shown that young tissue and old tissue behave differently in the presence of both non-host specific and host specific toxins. Neither of the toxins used, however, were cercosporin.

A new in vitro assay was employed to measure the inbreds' resistance to cercosporin. Prior to this, however, an in vivo assay was attempted. This assay gave ambiguous results as lesions developed for both the cercosporin treatments and some of the DMSO control treatments. Due to the crude methodology of this assay, even penetration of both cercosporin and DMSO into the leaves was not achieved. Therefore, this assay lacked reproducibility and was no longer used. The assay which gave the most reproducible results was the ion leakage assay.

The ion leakage assay was more sensitive than the dark CO_2 fixation assay as it measured the presence of ions in solution against a near zero background. The dark CO_2 fixation assay, on the other hand, measured a decrease in the rate of $^{14}CO_2$ fixation against a high background. A problem with variability between repetitions of the same treatment still existed, however, but not to such a high degree. The source of the variation was the initial conductivity of the water in the vials. The conductivity of the water in each vial was low and consequently very sensitive to small changes in the number of ions present in solution. Ions could enter the solution from the glass walls of the vials [illegible] fallen into the

vial. The result is that vials from the same treatment group had different time zero conductivities. Despite these differences, the reproducibility of this assay was good.

Concentration curves were generated to find a cercosporin concentration which resulted in a significant increase in conductivity compared to the increase of the controls. This would allow inbreds resistant to cercosporin to be easily detected in contrast to the susceptible inbreds. Ten micromolar cercosporin was chosen because it was an adequate toxin concentration to give a significant increase in conductivity for all four inbreds tested (Figs. 3-7). Using this concentration a total of thirteen inbreds were tested to see if any of them were more tolerant to cercosporin and to compare these results to their Grey Leaf Spot disease resistance scores.

Two of the inbreds were tested using tissue from plants 4 to 6 weeks old as well as from plants 12 to 15 weeks old to determine whether the plant's level of toxin resistance was affected by tissue age. The younger tissue showed a slight decrease in tolerance for the toxin. Since Cercospora zeae-maydis normally infects older plants this increase in toxin susceptibility of younger tissue is probably not significant. The inbreds do not lose any protection against the effects of cercosporin with age, but perhaps gain some protection. This phenomenon is similar to what is seen when T-toxin from Helminthosporium maydis is used to induce ion leakage from corn tissue. The youngest leaves of corn seedlings in the three leaf stage were found to be more susceptible to the effects to the toxin than the older leaves. Older plants, however, showed the opposite effect with older leaves being more susceptible to the toxin than

linked to the content of free sterols in the cell's membranes (5). This variation in membrane composition may also effect the toxicity of cercosporin.

After screening all thirteen inbreds a weak, but positive, correlation between cercosporin resistance and Grey Leaf Spot disease resistance was established. This is shown by both the correlation between the slope of the experimental line and the GLS score, 0.5399, and the correlation between the mean increase in conductivity at T = 150 and GLS score, 0.5524 (Table 12). Since smaller conductivity values mean more resistance to cercosporin, whereas larger GLS scores mean more resistance to the disease, a negative correlation was expected. Also, none of the inbreds showed a highly significant resistance to cercosporin as shown by only a two-fold difference in the level of cercosporin resistance between the most and least susceptible inbreds. Some of the difference in the level of variation may be due to the age of the tissue. Since different inbreds mature at different rates and tissue age was not closely regulated, the tissue from some of the inbreds may have been a little older than from others.

In addition, the graphs of all thirteen inbreds are similar in shape (Figs. 8-20). The leaf slices were exposed to light at T = 15, and for each inbred a lag of approximately 15 minutes can be detected before marked increases in conductivity are observable. Cercosporin requires light to produce superoxide and singlet oxygen which lead to the destruction of the cell's membranes (10), so prior to being exposed to light the toxin is inactive. Presumably, after exposure to light a lag time occurs as cercosporin starts to generate toxic forms

increase as more radicals are generated, accelerating the rate of fatty acid oxidation allowing more ions to escape the cell as its membranes are destroyed. Since all thirteen inbreds have approximately the same lag period, the differences in cercosporin tolerance are probably not due to different levels of oxygen radical quenchers within the cell. If resistance to cercosporin was due to increased levels of oxygen radical quenchers within the cell, the lag time of a resistant inbred would be longer than a susceptible inbred's, since the resistant inbreds would be able to remove the oxygen radicals preventing the initiation of lipid peroxidation. Therefore, those inbreds which demonstrate more tolerance for cercosporin may be able to somehow slow down the process of fatty acid autoxidation.

Another possible defense mechanism against cercosporin would be the inhibition by the cell of the uptake of cercosporin. The effects of cercosporin when both irradiated (10) and non-irradiated (25) suggest that the toxin enters either the cell or the cell's membranes. Since cercosporin has polar functional groups attached to an aromatic hydrocarbon ring, it is logical to assume that it could enter the hydrophilic portion of a lipid bilayer. Differences in the levels of tolerance for cercosporin may be due to different cell membrane compositions among inbreds. Already there seems to be evidence that differing cell membrane composition may be responsible for differences in toxin tolerance between tissues of different age (5). A another defense mechanism against cercosporin would be the production of enzymes capable of destroying the toxin. The data does not support this defense mechanism, however.

In addition to controls which contained only DMSO and no toxin, a control which contained toxin but was never exposed to the light was run for each inbred. These controls did not show a significant increase in conductivity as compared to the experimental vials so they were included only once per inbred. For ten of the thirteen inbreds the dark control vials showed a slightly greater increase in conductivity than did the no toxin controls. As a result, cercosporin was thought to be able to change the conductivity of the solution by itself. Using an immersion electrode a change in conductivity was observed when cercosporin was added to a solution which did not contain plant tissue. In the presence of plant tissue, however, cercosporin did not cause a jump in conductivity. Possibly, cercosporin either combined with a by-product from the plant cells or was immediately taken up into the plant cells preventing it from changing the conductivity of the solution. Attempts to study this further were unsuccessful since the jump in conductivity in the absence of tissue could not be observed using the draw-up electrode. Since the assay was not able to reproduce the jump in conductivity when cercosporin was added to the solution in the absence of tissue using the draw-up electrode, it was concluded that the conductivity jump was an artifact of the immersion electrode.

An alternate explanation for the greater increase in conductivity of the dark toxin controls as opposed to the no toxin controls is that cercosporin has a small effect on plant cells even when non-irradiated. Other researchers have suggested that cercosporin has an effect on the ion transport of sugar beet leaf cells when non-irradiated (25). Some toxins, such as HC-toxin, actually

among inbreds, the same thing occurs in corn leaf cells. To better understand whether cercosporin has an effect on corn when non-irradiated, ion leakage assays could be performed in complete darkness. Also, experiments can be set up to demonstrate if it is possible for a Grey Leaf Spot infection to spread on plants kept in the dark for prolonged periods of time. In addition further work needs to be done to determine exactly what effects cercosporin may have on the conductivity of a solution in the absence of tissue.

Of the thirteen inbreds which were tested none supported the hypothesis that cercosporin resistance may be a means of preventing infection by Cercospora zeae-maydis. Testing of more inbreds, however, may uncover an inbred that shows cercosporin resistance. Conversely, it could be that those inbreds which are resistant to Grey Leaf Spot disease, but demonstrate an increased cercosporin susceptibility utilize a hypersensitive reaction to protect themselves from infection. Hypersensitive resistance involves a rapid cell destruction which effectively walls off the fungus from infecting more of the plant by creating a zone of dead cells. On the other hand, a likely possibility is that disease resistant plants may not even utilize a defense mechanism which involves cercosporin, but instead inhibit the fungus at some other stage of infection.

Another possibility which is worth noting is that the assay used may not accurately reflect the degree of resistance of the leaf tissue to cercosporin. Perhaps, the results obtained were artifactual. To further support the findings of this report another assay, such as staining cells for viability which might be more representative of actual cell death, could be run.

CONCLUSIONS

The relationship between cercosporin resistance and Grey Leaf Spot disease resistance was the inverse of what was originally expected. Therefore, it was concluded that resistance to cercosporin does not have a significant role in preventing Grey Leaf Spot infections. On the other hand, an acute sensitivity to cercosporin may help protect disease resistant lines through a hypersensitive type of reaction.

The ion leakage assay proved to be a reproducible means of measuring an inbred's sensitivity to cercosporin. The additional modifications made to the assay further increased the its reproducibility. These modifications also allowed the assay to be run in complete darkness. In addition, the ion leakage assay could be utilized to test varying compounds which may be able to protect tissue from the deleterious effects of cercosporin.

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